

Are fast reactors gone for good?

Japanese and French ambitions apart, the fast reactor business is in the doldrums. But it would be a mistake to suppose that it will never have a future.

FAST nuclear reactors may not yet be dead, but it is far from clear whether the business of building them is still alive. Last week's announcement from Japan (see page 575) that the first full-scale reactor of this type in Asia has been commissioned does not suggest that it will be quickly followed by others. And while the attempt by environmental groups in France to use the decision to restart Superphénix for drumming up generalized anti-nuclear sentiment may yet succeed (see page 576), no date has yet been set for a full-scale breeder to succeed the demonstration plant on the Rhône. Indeed, in Britain the experimental breeder reactor that has operated successfully at Dounreay on the north coast of Scotland for 20 years was closed down permanently earlier this year. It is an open, but an interesting, question whether the business will ever revive.

There are several difficulties, of which the most immediate is technical. Breeder reactors, it will be recalled, necessarily generate thermal power at very high density and so must have efficient cooling systems. From the outset in the 1950s, liquid sodium has been the candidate coolant of choice. In nearly half a century, much has been learned of the properties of this material in bulk; it does not, for example, catch fire in air as might be expected. But experience has also shown that the facility with which liquid sodium can corrode welded joints in stainless steel heat exchangers (in which the primary coolant transfers its energy to pressurized water) is a perpetual headache, a source of shut-downs and of extra costs. It will be interesting to see whether the future yields a technical fix nobody has thought of yet. But nobody should count on that.

Economics

The economics of breeder reactors are, by comparison, a secondary problem. The far-from-eager demand for natural uranium which, after enrichment, is used in thermal reactors means that its price (after allowing for inflation) is lower than predicted even two decades ago. That in turn diminishes the benefit that breeders offer of yielding more fissile material than they consume. (The fissile material is not free; there is, of course, the associated extra cost, compared with thermal reactors, of building and operating them, of reprocessing the spent fuel and of extracting the fissile product.) On present technical understanding and with the present price of uranium, fast reactors capable of breeding extra fuel would generate electricity only at a greater cost than thermal reactors. So much is agreed even by those keen to see more

thermal reactors built. The imponderable is how soon the demand for natural uranium will increase again, as it must do if ever the threat of global warming is taken seriously. On past form, the World will begin to take energetic action only when the evidence for global warming is not merely incontrovertible, but plain for all to see and probably a daily nuisance as well. Delay, of course, will be expensive, but the costs of remedial measures or of adaptation (if they are affordable at all) will then be shouldered eagerly. When that happens, the fast reactor will come into its own again.

Safety

There remains the issue of safety, which has two aspects in this connection. Plutonium, the fissile product of uranium, is especially toxic because of the special affinity of all its several isotopes for bone, and the high specific radioactivity of some of them. (Interestingly and disappointingly, the thorium cycle, whose fissile product is the uranium isotope ^{233}U , and which is potentially a fuel for fast breeders, has never been given a serious trial.) Fuel fabrication obviously entails special risks. So does the reprocessing of spent fuel. But the Japanese will no doubt tell us how that is best accomplished.

The far more telling objection to the safety of fast breeders is that their fuel, by definition, is fissile and so suitable for making bombs. How, if the world were to depend on fast breeders as a substantial source of energy, could it be arranged that the fissile material would not find its way into nuclear bombs deployed by recalcitrant governments or even terrorists? The difficulty with that argument is that fast breeders are not the sole source of fissile material. Enrichment and reprocessing plants (which are not going to go away) already yield nuclear explosives whose use needs to be safeguarded. This newspaper has argued long and hard that the mere existence of such plants (not to mention the fissile material taken from dismantled superpower weapons) requires an international regime for safeguarding and approving proper uses for stocks of fissile material. Fast breeders would increase the quantities to be handled, but would not complicate the principle that must be grasped.

Meanwhile, there can be no doubt that the question of the revival of the nuclear power industry is a question of "WHEN", not "WHETHER?". We find ourselves in strange circumstances. The belief that the accumulation of greenhouse gases in the atmosphere will change the climate uncomfortably, if not (in the long run) for the worse, is widely shared, but rarely



translated into a sense that steps taken now to fend off that happening will be more economical than the steps it will be necessary to take at a later stage if a decision is delayed. Nuclear power engineers now being put out of work may comfort themselves that there will soon come a time when the world is crying out for their successors. It is not beyond the bounds of possibility that some of them will be employed in building fast reactors. □

Patents for eugenics

Eugenics is both unpopular and widely practised; will it never contribute to the human condition?

EUGENICS has a bad name, but is the practice of this supposed black art as despicable as commonly supposed? The origins of the bad name are mostly historical and largely, but not exclusively, to do with Adolf Hitler. His position, unforgivable and unforgiven, was simple: many people (Jews, Gypsies, the congenitally disabled and even miscellaneous critics) are genetically undesirable and, for that reason, should be done away with. The other side of that coin, the goal of excavating from a whole people's genetic endowment the embodiment of a 'pure' and inherently 'superior' race of human beings was known, even in the 1930s, to be genetically unattainable and replete with the disastrous potential of allowing a whole repertoire of recessive genes to come into their own. Even on the basis of the little then known about the working of the human genome, that argument was part of the liberal counterattack on the English eugenics movement of Edwardian times.

Hitler has now been thoroughly repudiated, especially in Germany (the criminality and noisiness of neo-Nazis notwithstanding). But has the repudiation been too thorough? That question now arises in an arcane form, in relation to a patent application submitted to the European Patent Office (EPO) on behalf of researchers at the University of Pennsylvania who have outlined a technique for arranging that the mammalian testis should be able produce only genetically engineered spermatozoa. In brief, the scheme would go beyond the essays in gene therapy now being attempted, and which are aimed exclusively at correcting congenital errors of metabolism by altering the genetic constitution of somatic cells in the individuals affected. Instead, it would manipulate the germ line of affected animals. A groundswell of opinion is building up that the EPO should refuse the application on the grounds that it offends against the rubric in EPO's charter that forbids patents for inventions that offend against "morality and public order".

This has all the makings of a splendid irrelevance. In the first place, the technique outlined is evidently aimed at the production of domesticated animals with desired genetic properties; the authors say that they included human beings only for completeness. Second, it is more a scheme than a tried technique, and may not qualify for protection on those grounds. Third, it is unthinkable that any human family, however anxious to rid itself of some unwanted version of a

gene, would think of subjecting any one of its males to such a procedure when the risk of misplacing the gene in the genome must be very great — and the risk of doing more harm than good to offspring must be very high. And finally, it should not be part of the function of the EPO (although legally it is) to adjudicate on matters of morality and public order.

But what will happen when it is possible confidently and safely to manipulate genes in the cells of the human germ line? And should not the first patent application in the field be rejected so as to discourage those who will later seek protection for germ-line manipulation that could be safely used for human eugenics? That is how the argument goes, but it is inapplicable for several reasons. The least of them is that even the existing techniques of amniocentesis followed by abortion in the circumstances allowed by law constitutes eugenics of a mild sort; the offspring of those born after screening for an unwanted allele can be sure that they will not inherit that allele from the parent concerned. That may not be a means of changing the genetic constitution of a whole population quickly or decisively, but it is a eugenic procedure that greatly assists (and is welcomed by) many modern parents. Democratic governments, which cannot compel putative parents to take advantage of these techniques, also have a responsibility to encourage their use. Why should it draw a line (and where) between that familiar practice and germ-line manipulation if it could be safely practised?

The second and more important reason why EPO should resist the pressure to which it is now being exposed is that there is already national legislation (and the prospect of a European directive) where its writ runs that prevents genetic manipulation except under licence. If it took ten years (in the United States) to get the first somatic-cell gene therapy under way, how much longer will be the debates in the regulatory committees about proposals for the manipulation of germ-line cells and the organs in which they lie? And since the grant of a patent does not exempt its holder from the structure of national laws, what sense does it make to saddle EPO (and national patent offices in Europe) with responsibility for deciding moral questions that properly rest with national parliaments?

Arcane though its present origins may be, the question of whether techniques for manipulating the human germ line should be patentable is an important one. As things stand, there is no technique that can be safely used, whence the standard response of geneticists in the field, "We will never touch it!" But that would have been the response of most professional people half a century ago to the prospect, now a welcome reality, of amniocentetic screening (or even the genetic diagnosis of blastocysts). Can one be sure that germ-line manipulation can never be safe? Or that, if and when it is, putative parents of that future generation will shrink from it as energetically as they do now? In the circumstances, open-mindedness is the most valuable asset, but those who call themselves "ethicists" should bend their minds to the definition of the circumstances in which national eugenic policies conflict with the rights of people to be individuals. That, of course, will turn out to be an enquiry not in genetics, but into prejudice, of which there is too much. □

Hubble upgrade review stirs concern over impact of NASA budget cuts

Washington. The US National Aeronautics and Space Administration (NASA) is considering whether to scale back its plans to maintain and upgrade the Hubble space telescope, only months after a successful repair trip rescued the project and gave the agency its biggest public relations boost for several years.

Wesley Huntress, NASA's associate administrator for science, has told scientists at the agency and outside astronomers to postpone proposals for an advanced camera for Hubble — due to be fitted on the platform during a 1999 maintenance flight — pending a review covering the Shuttle missions planned to Hubble in 1997, 1999 and 2002.

The postponement comes only three weeks after NASA had issued its first 'announcement of opportunity' for the advanced camera. It has angered scientists, and has led to speculation that budget pressures on the agency may lead it to sandwich the first two trips into one mission in 1999, when a Shuttle mission will be essential to raise Hubble's orbit and prevent it falling to Earth.

Such a course of action would mean a two-year stretch out of the construction of two new instruments, the Near Infrared Camera Multiple Object Spectrometer (NICMOS) and the Space Telescope Imaging

Spectrograph (STIS), both due to be launched and fitted in 1997. It might also lead to a decision to shelve the proposed advanced camera, which scientists say is needed to exploit fully the newly improved resolution being achieved by Hubble. It would, however, save NASA the costs of the shuttle launch in 1997.

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Will impact of last December's repair mission (simulated here) be kept up?

Ed Weiler, the programme scientist for Hubble, is seeking to reassure scientists that the review in itself does not mean that the programme will be radically changed. "I cannot predict the outcome of the review," he says. "I don't think anyone should take the postponement too much to heart."

But some astronomers predict that the process will in itself damage the scientific activities of the telescope. "Everyone is concerned, and it's hard to stay focused on your work when there is this kind of turmoil," says Jim Crocker, head of advanced programmes as the Space Telescope Science Institute at Johns Hopkins University in Baltimore, Maryland, which manages the Hubble science programme for NASA. "We've been reviewed to death on this programme. At some point you have to set your course and proceed on it."

NICMOS and STIS were halved in scale in previous reviews, and will now cost about \$70 million each. The principal investigator of NICMOS, Rodger Thompson of the Steward Observatory at the University of Arizona, Tucson, says that scientists should not be too concerned. "NASA is facing a reduced budget and I'd expect them to evaluate each of their options carefully," he says. "At the moment we are not hesitating. You cannot let these things divert you from the matter in hand."

But uniquely among NASA missions, Hubble is supposed to be regularly maintained and updated over its planned 15-year life. Advocates of this approach are calling on scientists to defend a concept that, they say, enables the potential of the maintained craft to be fully exploited.

More than 60 per cent of Hubble's total budget — twice that of some other programmes — goes on science. Frank Cepollina of NASA's Goddard Space Flight Center at Greenbelt, Maryland, Hubble's servicing manager, says scientists should rally round the concept of not throwing away assets. "Economically it is what makes sense, and it is what our customers — the public — understand."

But NASA is having to work out how to absorb impending budget cuts without killing any of its biggest programmes. John Pike of the Federation of American Scientists predicts that Congress may cut as much as \$400 million more from NASA's \$14-billion budget for next year, on top of the \$250-million cut already proposed by the administration.

"I don't think anyone [at NASA] has a clue where that is going to come from," says Pike. Big programmes, including the space station, the Cassini mission to Saturn and the aeronautics programme, have support from key lobby groups, says Pike; "what's left is \$10 million here and \$20 million there". In scurrying after cost savings at Hubble, Pike says, Huntress "is in no different position from anyone else".

Colin Macilwain

Ocean experiment is put on hold

San Francisco. The Advanced Research Projects Agency of the US Department of Defense has decided to hold back funding for part of an experiment to measure global warming in the ocean, pending a hearing being held in response to claims from environmentalists that the experiment could be harmful to whales and other wildlife.

The study is known as Acoustic Thermometry of Ocean Climate, and has been designed by the Scripps Oceanographic Institute. It is planned to measure changes in ocean temperature by sending sound waves from transducers deep in the water near Monterey Bay, California, and in Kauai, Hawaii.

The experiment became embroiled in controversy last month when a group of marine biologists claimed that the sound waves — coded signals of up to 195 decibels to be transmitted at between 60 and 90 Hz for 20 minutes every four hours — would disrupt the habitat of whales, potentially damaging their health and perhaps even causing their death (see

Nature 368, 485; 1994).

The halt affects only the California part of the experiment. But Scripps cannot continue until work at both sites is approved. Many prominent experts in marine mammal biology and bioacoustics have come to Scripps' defence, arguing that the claims of potential harm have been grossly exaggerated.

Most of the criticism has come from Hal Whitehead and his wife, Lindy Weilgart of Dalhousie University, both experts in sperm whales. Whitehead and Weilgart argue that the noise transmissions could seriously disrupt the habitat in which the whales live; their critics claim that they have become "too close to their animals".

Kenneth Norris of the University of California at Santa Cruz, a leading cetacean biologist, has described the experiment as "a brilliant solution to a vexing problem". But Andrew Forbes, deputy director of Scripps, says the delay will provide a useful opportunity to "educate the public".

Joel Shurkin

Genetically altered tomato set to get the green light

Herndon, Virginia & San Francisco. A rot-resistant, vine-ripened tomato seems likely to become the first genetically engineered food to go on direct sale in the United States, after an advisory committee to the US Food and Drug Administration (FDA) concluded at a public meeting last week that there are no outstanding safety issues to be resolved.

The conclusion has been widely welcomed in the biotechnology industry as a valuable boost to public confidence in genetically engineered products, particularly after the controversy surrounding the introduction in February of Monsanto's genetically engineered bovine growth hormone, BST.

As a result, the tomato, produced by Calgene of Davis, California and due to be sold under the brand name *Flavr Savr*, could be in grocery stores by summer. But some critics are still arguing that final clearance should be withheld until outstanding questions concerning issues such as pre-market notification, labelling and concern about allergenicity (the potential for causing an allergic reaction) are resolved.

The three-day meeting was held at the request of FDA Commissioner David Kessler to determine whether the process already used by the agency to evaluate the safety of new whole foods such as the *Flavr Savr* tomato was adequate.

Calgene was not required to ask for approval from regulators, as the FDA decided in May 1992 that foods developed through biotechnology are not inherently dangerous

Last week's meeting was held less to meet a specific regulatory requirement than to address public confidence in genetically engineered products. As such, John Bedbrook, executive president of DNA Plant Technology (DNAP), based in New Jersey, says it is likely to prove "pivotal" for the agricultural biotechnology industry.

The Foundation on Economic Trends, an activist group based in Washington that has consistently opposed genetic engineering, has already threatened to organize a boycott of the *Flavr Savr* tomato.

But most committee members felt comfortable about approving the tomato for sale. Gustaaf de Zoeten, for example, professor of plant pathology at Michigan State University, said he was satisfied that all the necessary safety considerations had been addressed. Arguing against any additional delays — it had been about 5 years since Calgene first undertook field testing of its *Flavr Savr* tomato — de Zoeten said the company should not be held hostage until the more general questions are resolved.

At the same time, a few of the advisory committee members — and several of the public participants in the meeting — expressed concern at the failure of last week's meeting to address on some of the broader issues involved.

Jane Rissler, staff scientist at the Union of Concerned Scientists, said that FDA should respond to the thousands of negative comments on its biotechnology food policy before approving any genetically engineered whole food. To do otherwise, she said, would be "premature".

Others noted that, while Calgene was willing voluntarily to submit safety and environmental data on its *Flavr Savr* tomato to intense public scrutiny, other companies may be less cooperative. Calgene also plans voluntarily to label its product. Indeed DNAP, which expects to enter the market early next year with its own genetically engineered tomato, says it has no plans to submit its product to the FDA for scrutiny — or to label it in any special way.

The use of antibiotic resistance genes as selectable markers — the kanamycin resistance gene is used in the *Flavr Savr* tomato — was also called into question, particularly in view of the reported build-up of antibiotic resistance.

Whether the agency will require some form of pre-market notification is "under active consideration", said Kessler. At least for the time being, the FDA is likely to implement a policy requiring growers and manufacturers to notify the agency of their intention to sell genetically engineered foods.

Diane Gershon and Sally Lehrman

Columbia aims to raise \$100m a year from new ventures

Washington. Columbia University, which earns \$31 million a year from licences based on research carried out by faculty members, more than any other US university, plans to use some of this income to fund a new venture to encourage close collaboration between its staff and industrial partners.

The Columbia Innovation Enterprise (CIE), to be launched in July, is a "bridging organization" to take ideas originating in the university and adapt them to industrial use, according to Michael Crow, the vice-provost. Through a strategy of "innovation enhancement", CIE will help Columbia researchers to modify products developed in the laboratory, from software to biological cells, and make them "more palatable for outside consumption", says Crow.

The university plans to publicize the availability of these and other research products through a computerized information service. According to Crow, CIE will engage in a "conscious, planned effort" to develop new commercial enterprises in such areas as biotechnology and manufacturing.

In addition, the university plans to establish a new network using telecommunications to allow companies all over the world to work with Columbia researchers. The purpose of this network, says Crow, is to move away from isolated, *ad hoc* associations. The university will test a pilot version of the network next year in Asia and the United States, and hopes to have an operational network early in 1996.

CIE will respect the same conflict-of-interest rules covering faculty-industry cooperation as those observed by its predecessor, Columbia's Office of Science and Technology Development. Faculty members will not be allowed to hold equity positions in companies for which they are doing research, and only "minor delays" to protect proprietary information will be allowed when publishing research results.

Although most royalty rights are retained by the inventor or discoverer at Columbia, some 140 licences are held by the university itself. In such cases, 25 per cent of the royalties go to the individual who carried out the research, 25 per cent to the university, and the rest is split between the department or research group and the body which sponsored the original work.

This year Columbia will earn \$31 million in licensing fees, based on product sales worth \$2.8 billion from inventions and discoveries made at the university. This puts Columbia alongside Stanford University as the top two money-makers in this area. Crow says that CIE's goal is to generate an income of \$100 million a year after nine years.

Tony Reichhardt



and will not normally require pre-market approval unless they contain substances new to the food supply or known allergens.

But the company had requested a safety review in order to meet potential criticism from consumers. Its new tomato has been modified to suppress the production of an enzyme that causes it to go soft, giving producers more time to let tomatoes ripen on the vine before being picked.

Japan's fast reactor heads for the slow track

Tokyo. A year and a half behind schedule, and surrounded by protesters, Japan's multi-billion-dollar prototype fast-breeder reactor Monju reached criticality for the first time last week. But it did so amid growing signs that Japan is likely to delay the introduction of commercial breeder reactors, initially planned for 2010, beyond even its revised target of between 2020 and 2030.

Construction of Monju was completed in April 1991, at a cost of ¥600 billion (US\$6 billion), and the reactor was scheduled to reach criticality in October 1992. But problems with production of the fuel rods pushed back that date three times. It will still be at least another year before Monju starts producing electricity, and the plant will not reach its full capacity of 280,000 kW until December 1995.

Japan launched its fast breeder programme several decades ago, at a time of widespread enthusiasm for such reactors. Since then, the United States has abandoned its programme, Britain and Germany have frozen theirs, and France's Superphénix reactor has been plagued with problems (see page 576). But Japan has stuck doggedly to its plans, although with delays.

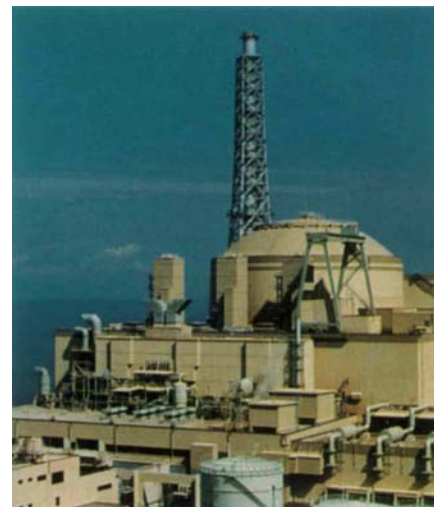
Nevertheless, in the mid-1980s, Japan put back the target for commercial breeder reactors from 2010 to between 2020 and

2030. Now even the new dates are looking increasingly unlikely; in January, the Federation of Electric Power Companies decided to postpone by several years — to around 2005 — the construction of a demonstration reactor planned to follow Monju.

Growing international concern about Japan's accumulation of the plutonium needed to power breeder reactors could also result in delays. Japan's first nuclear fuel reprocessing plant is due to begin operation in Rokkasho, a remote village in northern Japan, in 1998. But in February, unnamed officials were quoted in newspaper reports as saying that plans to build a second reprocessing plant for producing plutonium are likely to be postponed by 20 years (from 2010 to 2030) to prevent the accumulation of a plutonium surplus.

More recently, officials of the Ministry of International Trade and Industry have been reported as saying the government will have to scale down recycling plans on the grounds that having a major capacity to produce plutonium is a threat to world peace.

Last Saturday (9 April), suspicions of a likely delay were reinforced by a separate report from the Japanese Broadcasting Corporation that the Atomic Energy Commission has decided to postpone construction of the second reprocessing plant. But this will



Monju reactor now critical.

not be officially announced until the commission releases its new long-term nuclear power policy this summer.

Officials of the Science and Technology Agency in charge of Monju and nuclear fuel recycling admit that the commission is likely to announce delays on several fronts. They say the target of introducing commercial fast reactors between 2020 and 2030 is "under review".

David Swinbanks

French lab drops US deal over access to DNA bank

Paris. The researcher at the centre of a dispute over the future of a DNA bank collected from more than 800 French families and based at the Centre d'Etudes du Polymorphisme Humain (CEPH) in Paris (see *Nature* 368, 175; 1994) was claiming victory last week after the board of CEPH abandoned a proposed deal that would have given Millennium, a Massachusetts-based biotechnology company, exclusive access to the bank.

Philippe Froguel, whose group developed the bank, was fired from CEPH last month, after publicly opposing the proposed agreement. But matters have since been taken out of CEPH's hands by the government, which has set up a commission to decide how to regulate the commercial use of DNA banks.

High among the government's concerns is the threat that foreign companies may gain control of research "made in France"; the scientist heading the commission, Pierre Louisot, is also responsible for protecting France's "scientific heritage" at the biomedical research organization, INSERM (see *Nature* 367, 587; 1994).

The board of CEPH has now decided, after what is reported to have been a heated debate, to abandon the proposed deal with

Millennium. Froguel says that CEPH has also assured him that he can continue working at the centre until he finds another laboratory, and that he will be able to take a copy of the DNA bank with him when he goes.

Froguel has asked the commission to give him exclusive access to the bank, in collaboration with research groups of his choice, for one or two years before the bank is made publicly available.

Froguel says he is considering a move to the Cochin hospital in Paris. But another possibility would be to set up a laboratory in the United Kingdom.

Meanwhile Hoffmann-La Roche, the Swiss-based pharmaceutical company, has signed a US\$70 million deal with Millennium, to develop therapeutics for obesity and Type II diabetes based on research into the genetics of the diseases. Most of the US\$70 million will pay for research. But Roche will also take an undisclosed equity stake in Millennium, a company set up last year with US\$8.45 million of venture capital.

Roche says that its main interest in the deal is to develop technologies that "open the doors" to other therapeutic areas in the long term. It has already taken a major equity stake in Genentech, in a similar move to buy into know-how.

Under the terms of the deal, Millennium will carry out screening of cDNAs, identify mutant genes in mouse models and isolate disease genes by positional cloning. The company will also work out what the genes do, and design therapeutic agents.

Roche will get exclusive worldwide rights to small molecule therapeutics developed by Millennium. It also gets exclusive rights to antisense, protein, and gene therapies outside North America, while Millennium will retain these rights in North America, subject to Roche's option to co-promote.

Initial plans envisaged that Millennium would have provided access to the DNA banks at CEPH. Indeed, Michael Steinmetz, vice-president and group director for preclinical research of Roche, emphasized the importance of the CEPH DNA bank to the proposed deal with Millennium in a letter written earlier this year to Jean Dausset, the president of CEPH.

Sherry Reynolds, a spokeswoman for Millennium, says it has a "broad strategy" for gaining access to DNA from several groups. The company may also get non-exclusive access to the French bank, she adds, but this will depend on the outcome of the French government commission's discussion, expected in June.

Declan Butler

Palace coup may lift status of Biosphere 2

Washington. Dramatic events at the giant hermetically sealed greenhouse known as Biosphere 2 in the Arizona desert could lead to enhanced scientific credibility for the controversial facility, according to scientists close to the project.

Two weeks ago, Ed Bass, a Texan billionaire who has provided the financial backing for the \$150-million project, sacked the entire management team, led by Margret Augustine and John Allen, which conceived Biosphere 2 in 1984 and have run it ever since.

Bass issued a statement saying that the team had failed to implement sound financial controls and business practices. But it has also emerged that Bass was recently advised by scientists who have worked with Biosphere 2 that it could work better — and possibly attract federal funding — if it adopted a more focused scientific programme.

The sudden departure of Augustine, Allen and other 'true believers' in the project, if upheld by the courts, leaves the future of the facility wide open. The recent chain of events has brought the project's credibility to an all-time low. But some biologists think the departure of the founding individuals could help the facility to realize its full potential.

The 3-acre greenhouse is described as the largest closed ecological system in the world. Much of the early work carried out in Biosphere 2 has been derided by the scientific community for its lack of rigour. But the facility itself is seen as having great potential for the investigation of key ecological questions, such as the interaction between plants and carbon dioxide.

Wallace Broecker, a geochemist at the Lamont-Doherty Earth Observatory in New York State, says that a number of scientists who have worked with Biosphere 2 feel it could be made into a good facility. "It should

be converted to focus on the study of carbon dioxide and plants," he says.

"They need a much more disciplined approach," says Broecker. He suggests that Biosphere should commission leading out-

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Happier times: Abigail Alling, a former inhabitant now charged with trespass and criminal damage.

side scientists to produce a new research plan. Broecker thinks that it would then be in a position to attract funding from government agencies, as well as to increase its attraction to tourists. Similar views were recently conveyed in some detail to Bass, although their effect is unknown.

As chairman of Space Biospheres Ventures (SBV) — the company that owns Biosphere 2 — Bass has taken court action in Texas to dissolve a joint venture with a company based in Oracle, Arizona, and headed by Augustine and Allen, that operates the facility.

Pending completion of the action, the court has granted an injunction barring Augustine, Allen and four senior colleagues from the site, and has appointed a temporary receiver, Martin Bowen, to run Biosphere 2.

The anger provoked by Bass's action became clear when two more staff members suspended by Bowen — former Biosphere inhabitants Abigail Alling and Mark Van

Thillo — allegedly smashed five air seals on Biosphere 2 and opened some of its doors.

Alling and Van Thillo announced publicly that they had done this in order to safeguard people working inside Biosphere 2. But both were subsequently arrested and charged with burglary, trespass and causing criminal damage.

Gonzalo Ariza

Chris Helms, a spokesman for SBV, says that Biosphere 2 will function as usual under Bowen's management until the case, due to be heard by a court in Fort Worth on 19 May, is resolved.

Meanwhile, the research director, Jack Corliss, maintains that Biosphere 2 is continuing as a serious scientific project. "This is a change of management," he says. "It is not going to effect the evolution of Biosphere 2 into a top-notch research facility."

Corliss says that the break-in at Biosphere 2 resulted in the loss of about 15 per cent of the air inside the facility. But he claims it will not damage experiments because the nature of the change has been closely monitored.

Corliss also says he has not been asked to leave, and has no plans to resign. But some outside scientists are already speculating that Corliss's departure might be part of any effort to put the project on a new scientific footing.

Further evidence of turmoil at Biosphere 2 emerged last Friday when Norberto Alvarez-Romo, mission control manager and a member of a team of seven working inside the enclosed facility, was replaced by another manager.

A statement said Alvarez-Romo had left Biosphere 2 for a "family emergency", and to play a key role in mission restructuring". The statement contained no comment from Alvarez-Romo; previous statements from SBV had said that all of the crew had agreed to stay inside the facility.

In the longer term, Bass faces three main options if he wishes to cut his losses at Biosphere 2. The first is outright closure, which would write off almost all of his investment. Helms says categorically that the facility will not be shut down.

The second option is to make the venture more brazenly commercial, opening it as a kind of theme park. The third is to bolster the project's credibility by reintroducing a proper structure for the organization and peer review of science at the facility.

Helms says it is too early to comment on these options, but admits that restoration of a panel of outside advisers — the last one resigned *en bloc* last year, when it was not consulted on Corliss's appointment — is "possible".

Colin Macilwain

Protest march targets French reactor

Paris. Chanting "unplug Superphénix", about a thousand people set off last weekend from Creys-Malville, the site in southern France of the fast breeder reactor, on a four-week march that may herald the rebirth of the antinuclear movement in France.

Opposition in France to nuclear power has been muted in recent years, compared, for example, with the mid-1970s when a demonstrator was killed at Creys-Malville during running battles between the police and more than 20,000 protesters.

One reason is that opponents have been denied targets likely to rouse popular support. The government shut down Superphénix in 1987 following repeated leaks in its sodium cooling circuits, and

imposed a 15-year moratorium on the deep-storage of nuclear waste in 1991.

But Greenpeace and other environmental organizations have now seized the opportunity presented by the government's recent decision to restart Superphénix to use the reactor as a rallying symbol. The greens' main goal is to influence France's first national debate on future energy strategies, which will take place next month in the National Assembly.

The protest march will end on 8 May at Matignon, the prime minister's office, after stopping off at various nuclear plants on the way to Paris and commemorating the eighth anniversary of the Chernobyl meltdown on 26 April.

Declan Butler

India agrees to new terms on rocket contract with Russia

New Delhi. A controversial deal over cryogenic engines between India and the former Soviet Union has been renegotiated, ending a long period of suspense over the fate of the geostationary launch vehicle (GSLV), India's biggest rocket which is designed to launch commercial satellites into geostationary orbit.

The original contract, agreed in 1991, involved the supply of two cryogenic upper stages for the GSLV, together with the technology for local manufacture in India. But the deal had been hanging fire since the break-up of the Soviet Union, as Russia had come under pressure from the United States to cancel it (see *Nature* 364, 371; 1993).

U. R. Rao, the chief of the Indian Space Research Organization (ISRO), announced last week that a renegotiated contract between the ISRO and the Russian space company Glavkosmos has now been approved by both governments.

The new terms will be welcomed by US officials, as Russia will not transfer to the Indians the know-how for building cryogenic engines. The United States had feared that India might use this technology for making military missiles, but had not objected to Russia selling assembled engines.

Under the new contract, India will receive four ready-to-fly cryogenic upper stages, and two 'mock-up' versions, for the previously agreed price of \$80 million. In addition, ISRO has an option to buy up to three additional cryogenic engines at a cost of \$3 million each.

Overall, says Rao, India will receive seven cryogenic stages, with the first delivered in June 1996, to be followed by one every six months. ISRO is planning the first test launch of the GSLV powered by the Russian engine to take place in 1997.

The deal will provide much-needed capital for the Russian space company, which has already received more than half of the sum agreed in the contract. But ISRO has lost out. "We went in for the contract to get hold of the technology, not just ready-made engines," says one top scientist involved in the organization's \$120 million project to develop an indigenous cryogenic engine.

With the GSLV design already frozen, the ISRO project will now be constrained by the fact that the indigenous engine must have the same specifications as the Russian engine. But Rao remains confident that ISRO's own engine will be ready for testing by 1998 at the latest. To achieve this, ISRO hopes to take advantage of the lengthy training that its engineers have already received in Russia, as well as the design drawings which were obtained under the original contract.

K. S. Jayaraman

Study confirms AZT's lack of prophylactic effect

London. Azidothymidine (AZT), otherwise known as Zidovudine, is not an effective prophylactic of AIDS when used in people infected with human immunodeficiency virus (HIV), but in whom symptoms of AIDS have not yet appeared.

That is the final conclusion of the so-called Concorde study mounted by Britain, France and Ireland in 1988 in the wake of the decision by the US Food and Drug Administration (FDA) to license the prophylactic use of AZT.

A preliminary report on the Concorde trial, foreshadowing the final conclusion, was published last year (*The Lancet* 341, 889–890; 1993). As a result, the conclusions of last week's publication (*The Lancet* 343, 871–881; 1994) have been partly anticipated by both physicians and the stock-markets.

But the final report should quell controversies over the apparent disagreement between the Concorde trial, to which 1,719 symptomless people with HIV infection were recruited, and a handful of smaller trials, some of which suggest that the early use of AZT could be beneficial.

The Concorde Coordinating Committee now says that its conclusions are consistent with those of the earlier smaller studies — notably in the United States and Australia — once allowance has been made for the smaller size of other studies, their short duration, or their use of the CD4⁺ cell-count as a proxy for clinical improvement.

On this last point, the Concorde report suggests that early administration of AZT increases the concentration of CD4⁺ cells in the blood of symptomless patients, at least for the three-year period during which their condition was followed.

But the study found no significant difference between the death or disease rates among those given AZT at the outset of the trial, and those in whom its use was delayed until the appearance of symptoms.

Although originally designed as a double-blind study, in which neither patients nor their physicians would know whether they were in the control or study groups, physicians were allowed to put their patients on AZT if CD4⁺ cell-counts were low, or if symptoms of AIDS appeared.

Over three years, a total of 418 people — or a quarter of the total — became aware of their treatment for these reasons. But the final report insists that this does not undermine its conclusions.

The report also presents preliminary data on the side-effects associated with the administration of AZT. Apart from 6 people who died from medical conditions not obviously linked with treatment in the trial, 99 people in the treatment group, and 38 among those given placebos, stopped taking trial capsules for a variety of reasons, including gastro-intestinal difficulties, persistent headaches and declining haemoglobin levels.

John Maddox

Newspaper revives anti-HIV claims

London. The *London Sunday Times* has rediscovered AIDS. In an article headed "Conspiracy of Silence" on 3 April, the newspaper's science correspondent, Neville Hodgkinson, describes a "large and growing network" of "highly qualified" people who dissent from the "HIV hypothesis" and are willing to admit it.

The reference appears to be to the signatories of an open letter organized three years ago by Charles Thomas, now at the California State University at San José, appealing for a reappraisal. Kary Mullis, the inventor of the polymerase chain reaction (PCR) and last year's Nobel prizewinner in chemistry, one of the signatories, is quoted as saying that "the HIV hypothesis is unfalsifiable, and useless as a medical hypothesis".

The report also says that *Nature* has sought to dissuade the *Sunday Times* from "reporting evidence that HIV is probably not the cause of AIDS", and asserts that scientists "have to be careful not to rock

the HIV boat, which carries jobs, reputations and huge research funds".

It concludes with a statement by Hiram Caton, described as head of the school of applied ethics at Griffith University, New South Wales, that when "no vaccine will be forthcoming", scientists will "have to come to terms with the awful fact that the AIDS epidemic was a mirage."

A week later, on 10 April, the newspaper continued its campaign with a report of the results of the Concorde trial (see above). It quotes Peter Duesberg as saying that "When the time is ripe to say that AZT is detrimental, that it actually hurts, the interpretation will change again".

In the same issue, Hodgkinson describes a recent finding that 42 per cent of people attending an AIDS clinic in London had used amyl nitrite as an aphrodisiac within the previous month. The introduction to the article says that this "new evidence" shows that amyl nitrite is "an important cause of AIDS". □

Research boost blocked in Czech universities

Prague. Attempts to guarantee academic freedom in Czech universities have had the unintended effect of hindering efforts to boost research by encouraging resistance to government-backed reforms. Now the government is planning new moves to overcome the deadlock, and thus reach a goal that is widely accepted but is yet to be achieved.

In communist times, teaching and research were kept strictly separate. Universities were required to concentrate on the former, and lost most of their scientific activities to the research institutes of the Czech Academy of Sciences.

The post-communist regime — and, indeed, the whole academic community, both inside and outside the universities — is keen to reverse this situation. But attempts to stimulate the growth of research in universities have met with surprising resistance.

Paradoxically, the main stumbling-block has been the universities' newly established autonomy, guaranteed by a law passed in 1990. The universities are now strongly opposed to anything perceived as state interference in their affairs; and their autonomy

has allowed them to fend off pressure for structural reform.

For example, apart from the loss of some of the youngest and most talented academics to private enterprise, there has been no significant turnover in university staff since the new regime came to power in the so-called 'velvet revolution'. This is despite the fact that many appointments in communist times were made on political grounds rather than because of academic merit.

According to Stanislav Hanzl, rector of the Technical University of Prague and president of the Czech Rectors' Conference, the heads of university departments — who hold full responsibility for their staff — have accepted in principle that the competence of each academic should be reviewed. But in practice they have found it



Ondráček: optimistic for the future.

difficult to bring themselves to sack colleagues, particularly when they had no-one above them in the system to blame.

Last autumn the government, frustrated by the lack of progress, forced the issue by amending the higher education law to put all staff, including professors, on temporary contracts. Everyone will now have to re-apply for their jobs when their current contracts end (see *Nature* 366, 497; 1993). But this move has only increased resentment in the universities. "People are fed up with the constant pressure to compete with one another," says Hanzl.

Autonomy has also given universities control over their budgets. But despite a declared intention to improve support for research, the proportion of the budget allocated to this goal fell from 11 per cent in 1989 to around 6 per cent in 1992. In real terms the cut has been even bigger, as it has taken place at a time when the total budget for universities has fallen in value by nearly three-quarters.

Another problem is that university staff are often poorly qualified. Nearly half of university academics have no research degree, and it has been difficult to establish active research groups in universities without introducing new blood.

But some moves to achieve this have also been rejected. When the Czech Academy of Sciences, for example, was forced last year by budgetary pressures to cut back the work of its research institutes (see *Nature* 368, 386; 1994), it drew up a list of scientists it could no longer employ, but who, it believed, would be of value to universities.

The government established a Kcs100 million fund to help transfer these scientists into universities. But many universities felt they were being offered low-quality rejects — a charge which the academy denies — and as a result barely a quarter of the funds were used.

There is, however, new optimism that things may change soon as a result of recent moves by the government. This year, for example, university budgets include for the first time a component based on research output, rather than student numbers. At present, this component is only 10 per cent of the total budget; but it is planned to rise to about a third over the next few years.

In addition, a new law covering research in universities is scheduled to be introduced late next year. If passed, it will, for example, encourage greater exchange between academy and university scientists. Emanuel Ondráček, deputy minister in the department of universities and science, is already pleased about the way things are going. "Science has made big progress in the past six months in universities," he says.

Alison Abbott

'Ecotourism' plans come under fire

Moscow. Leading Russian scientists have criticized plans announced by the government of President Boris Yeltsin to encourage state-operated nature reserves to engage in commercial operations, for example generating income through admission charges for visitors and even allowing access for hunters for a fee.

Speaking at a meeting of the Russian Academy of Sciences at the end of last month, biologist Vladimir Sokolov called on his colleagues to oppose the government's plans, arguing that "ecotourism", while appropriate for national parks, could become a major threat to the nation's nature reserves.

Further criticism came from another member of the academy, Vadim Tihomirov, chairman of the academy's reserves committee, who said that he was strongly opposed to policies based on economic rather than environmental arguments. Excessive commercial pressures on the reserves could "lead to their death" he said.

Tihomirov quoted several examples of ways in which nature reserves are already being commercially exploited. In the Il'men mineral reserve in the Urals, for example, rare precious and semi-precious stones are being sold, while in the Caucasian biosphere reserve, a large area has been leased for 25 years to a private company producing plastic shoes.

Tihomirov said that such actions appear to ignore the extent to which the reserves are "eternal information resources" and facilities for measuring the Earth's "ecological temperature". They should be used for scientific research and investigation, "definitely not for commerce", he said.

The main target of the scientists' criticisms has been the new federation minister for environmental protection and natural resources, Viktor Danilov-Danilyan, who is an economist by training (his predecessor, Nikolai Vorontsov, is a prominent zoologist and evolutionist).

Danilov-Danilyan was responsible for the decree suggesting the commercialization of the reserves. But attempts to move in this direction are not new; the former Soviet leader Nikita Khrushchev, for example, tried to introduce such a policy, although his efforts were blocked at the time by the actions of the scientific community.

At the same time, Danilov-Danilyan has himself complained that the Russian government is trying to solve its economic problems at the expense of the environment. He told a recent meeting organized by his ministry that 1,700 draft programmes for improving the ecological situation in Russia are at risk due to insufficient financing.

Carl Levitin

Undue credit for supervisors

SIR — As a postgraduate completing a PhD and enjoying an excellent working relationship with my supervisor, I am concerned about the pressure that many departments, in many universities, are placing on research students to include their supervisors' names at the top of journal papers, regardless of whether the supervisors have made contributions to the authorship of the publications.

Although many research students choose to share the authorship of their research publications with their supervisors, it is by no means a universal practice. The last Research Assessment Exercise (RAE), however, has caused many UK university departments to sacrifice intellectual freedom for the sake of their positions in the research league. Under the terms of the last RAE, which sought to assess the research output of staff funded by the Higher Education Funding Council (HEFC), when a student publishes the results of his or her research, the department cannot include the paper in its list of publications unless a member of staff is a co-author.

In many cases, the contribution of a supervisor to a student's research is correctly acknowledged by co-authorship on papers. But the primary role of a supervisor is as an overseer and trainer, not as a co-worker. Such an input is more appropriately recognized in the acknowledgements at the end of a paper, together with the research student's grant number and the name of the sponsor. The last RAE, however, ignored the fact that the supervision of research students is, even without co-authorship, a significant and substantial part of the research output of university staff.

Desperate to boost their ratings, a number of departments are now insisting that supervisors ensure that their names appear on all of their students' papers. One consequence may be that supervisors will be able to veto any papers with which they are unhappy, whether they dislike the style of the science, the writing, or even the prospect of a research student receiving sole credit for work that may have been undertaken with only minimal supervision. The corridors of our universities already echo with rumours of supervisors who have stolen their students' ideas and data to publish as their own.

Surely the freedom to submit the results of their research for publication, with or without their supervisors' blessing, is an important prerogative for research students who have to demonstrate independence of thought to obtain their doctorate?

Indeed, is it not an act of scientific fraud to claim co-authorship for a research publication when one has not produced any of the ideas or data contained in a

paper? Even if no-one else cares, the editors of journals must be concerned by such a practice.

There is one very easy way for the HEFC to remove the cudgel that is currently being waved above the head of research students. In the next RAE, why not allow research students' publications to be included even when not co-authored by their supervisors? Without a change in the approach to the RAE, research students will continue to be regarded as mere pawns in the financial squabbling between university departments and the HEFC, rather than as the independent, wealth-creating scientists of tomorrow.

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In the eye of the beholder?

SIR — I was fascinated, but somewhat flummoxed, by the recent contribution by Perrett *et al.* addressing facial shape and judgements of female attractiveness (*Nature* **368**; 239–242; 1994). My problem is that in these days of political correctness, when we males are supposed to be fighting energetically against any biologically or at least constitutionally driven tendencies towards differential treatment of individual women (at least in the workplace), I am unsure whether to be challenged (the old me) or relieved (the new politically correct me) at being scarcely able to distinguish from one another the computer-reconstructed faces from within the two sets offered. In view of the severity of the effective selection process said to have resulted in, particularly, the third face of each set in relation to the first, is this not (or perhaps, am I as heterosexual male not) rather singular? While being able with study to see the differences, I certainly could not remember and attach differential names to faces within each set, and therefore cannot readily imagine developing differential feelings towards their owners on this basis alone.

Does my bluntness of conscious perception in this matter mean I am a victim of some high-order neurological condition, to be named in the appropriate textbooks alongside (if I remember rightly) prosopagnosia? I would welcome access to a small sample of other readers' personal findings. Alternatively, the theory might be that such differential perceptions can exist and exert their effects on behaviour, while by-passing altogether the consciousness of the perceiver. There are certainly

disturbing precedents for this, and yet the 'attractive' subset of primary data faces was selected by conscious processes. I suspect an important reality is that in real situations other differentials of women's total 'output' (appearance and behaviour combined), possibly through equally constitutionally mediated and thus politically problematic mechanisms, tend to have greater valency and to wipe out the contributions of all but extreme excursions from the norms of facial proportion to attractiveness ratings.

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Leprosy vaccine

SIR — In a News report describing a dispute between Indian scientists about candidate vaccines against leprosy¹, reference was made to work from our laboratory as establishing the identity of the vaccine strains. This is not accurate. In 1989 we published a report² describing the first repetitive DNA element in *Mycobacterium leprae* located adjacent to the *hsp 60* gene that had then recently been cloned. The paper shows that the insertional element was found only in *M. leprae* and not in six other mycobacteria, including the two Indian candidate vaccine strains with which we were kindly provided. Hence the repetitive element was not useful for studying polymorphisms in other mycobacterial species by DNA fingerprinting. The paper did show, however, hybridization bands to the *hsp 60* antigen found in all mycobacteria, and that the sizes of the DNA restriction fragments containing the gene were similar for the two Indian vaccine strains. However, this does not establish identity, as the number of bands in the patterns was small (only 2 or 3) and the *hsp* gene is highly conserved.

Since our publication, a number of mycobacterial genes have been sequenced, and several repetitive insertional sequences have been identified. With these new tools, it should readily be possible for an independent laboratory to establish definitively, by multiple parameter analysis, the identity or non-identity of the disputed strains.

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1. *Nature* **367**, 403 (1994).

2. Grossinsky, C. M., Jacobs, W. R. Jr, Clark-Curtiss, J. E. & Bloom, B. R. *Infect. Immun.* **57**, 1535–1541 (1989).

Italy's contribution to EMBL

SIR — As a founder member of the European Molecular Biology Organization (EMBO) and as an active Italian scientist, I wish to supplement what you say on the announced withdrawal of Italy from EMBL (the European Molecular Biology Laboratory, *Nature* **367**, 203 & 205; 1994).

The idea of creating EMBO originated during an international meeting I organized in Ravello in September 1963. I was then professor of physical chemistry at the University of Naples and director of a CNR unit of the Italian National Centre of Macromolecular Chemistry, whose president was Giulio Natta (who later won a Nobel prize for chemistry). A. Buzzati Traverso had founded in Naples the International Laboratory of Genetics and Biophysics (LIGB) and was an active partner in the foundation of EMBO.

The first step was the legal establishment of EMBO as a private foundation in Geneva, the second the promotion in 1970 through Swiss diplomacy of an inter-governmental structure, the European Molecular Biology Conference (EMBC), with the following aims: (1) to find ways to finance EMBO activities (courses, fellowships, workshops and eventually research grants) and (2) to create an European Molecular Biology Laboratory. An agreement was finally signed in 1973 to build EMBL in Heidelberg (not Nice as originally planned) with the help of the Federal Republic of Germany. EMBL grew quickly under the direction of John Kendrew (another Nobel prizewinner for chemistry).

The main philosophy of EMBO was not the promotion of biology as a specific discipline but of a 'new biology' in Europe, a 'science of science' as defined by J. D. Bernal.

A particular problem of Italian science and technology is the political partition (*lottizzazione*) of financial resources without regard to scientific excellence or reputation, which may explain the questionable claim of a "quota" in scientific (and not political) international institutions such as EMBO and EMBL. These should operate on the basis of an objective scientific evaluation irrespective of nationality. EMBL has so far achieved its main objectives and is rated among the world's leading laboratories.

It is true that CERN was taken as a model and we had some doubts about EMBL because molecular biology did not need a "big machine". But the moulding of qualified scientists (many of them now professors in Italian universities or directors of research centres) and the facilities provided by the outstations at Hamburg and Grenoble may be taken as just rewards for the financial contribution of

Italy which is based, presumably on some economic parameter. If some think that we spend more than we receive compared with other European countries, we must increase our scientific activities in this field. There is in fact no complaint about CERN. In any case, I think Italy's support for EMBO and EMBL is an "ethical" duty arising from the participation in their foundation of members of the Italian scientific community, as was also the case of CERN.

It is true that some new developing areas of 'new biology' are not properly covered by EMBL (neurobiology, theoretical biology, biomathematics and so on); these areas might be supported by small regional groups or by research grants regulated by EMBO and financed by the European Union.

Finally, I am confident that the international experience and common sense of the present Italian Minister of University and Science will provide a quick solution of the problem.

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Descent to tabloid *Nature*?

SIR — We note with disgust the latest descent of *Nature* towards tabloid science. We refer to the "cover story" of Vol. 368, Issue 6468 (17 March 1994) which relates to an article by Perrett *et al.* on "female attractiveness".

No doubt there is some case for the scientific analysis of human behaviour regarding the influence of appearance on mate selection and reproductive fitness. It is questionable, however, that such a study should be presented as focusing entirely on one sex, and that, as in this case, it should serve to perpetuate a deeply sexist attitude that objectifies women. Further, given that *Nature* has been a highly regarded international science journal, it must recognize the discreditable impact on research likely to result from emphasizing material of this kind. The public perception of scientific research as a worthwhile enterprise will be severely damaged if this editorial policy is continued.

As a protest, we shall not renew our subscription.

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Delaney and cancer

SIR — You justifiably say¹ that "Delaney must go", in reference to the Delaney Clause of the Food Additives Amendment, US Congress, 1958. This clause enacts that "no [food] additive shall be deemed safe if it is found to induce cancer when ingested by man or animals, or if it is found, after tests which are appropriate for evaluation of safety of food additives, to induce cancer in man or animal." The appropriateness of the tests was at the discretion of the Commissioner of Food and Drugs.

Supporters of the clause emphasize that no level of carcinogen is "safe". Of course, it has also become obvious that carcinogens "are present in many, perhaps most foods in a supermarket"².

The main obstacle to changing the clause is the one-molecule hypothesis of carcinogenesis. This hypothesis has been repeatedly affirmed by members of the National Cancer Institute (NCI), who have alleged that one molecule of diethylstilboestrol (DES) can induce cancer³. Arthur Upton (NCI director 1977–79) said in 1980: "Transformation of a normal cell into a cancer cell could conceivably occur with one molecule of a carcinogen acting on a single cell"⁴.

The "one-molecule hypothesis" sounds scientific, but it is not. If it were valid, we should all be cancerous from the millions of molecules of arsenic, cadmium and chromium in each one of our cells. Hutchinson⁵ has estimated the number of atoms of different elements present in each cell. The number for the heavy metals ranges between 10^4 and 10^6 atoms per cell. The arsenic content of a normal healthy human being is 4.4 mg, which is 9×10^{10} molecules of arsenic, a carcinogen, equal to about 10^5 per cell. Dinman⁶ pointed out the need to take stochastic considerations into account. He estimated that "a threshold for biological activity exists within a cell at 10^4 atoms": DNA repair mechanisms should also be considered in evaluating the effects of low levels of carcinogens. Also, a molecule of DES would have to compete for acceptor sites with a daily endogenous production per cell of 6,000 molecules of natural oestrogens, which are classified as carcinogens.

A retraction of the clause will have to depend on the perceived invalidity of the one-molecule hypothesis.

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- Nature* **363**, 287 (1993).
- Federal Register* **44**, 201 (16 October 1979), p. 59513.
- Jukes, T. H. *J. Am. Coll. Toxicol.* **2**, 147–160 (1983).
- Jukes, T. H. *Clin. Toxicol.* **17**, 475–476 (1980).
- Hutchinson, G. E. *Proc. natn. Acad. Sci. U.S.A.* **51**, 930–934 (1964).
- Dinman, B. D. *Science* **175**, 495–497 (1972).

Fresh start for Europe

SIR — The article by I. Barry Holland, "A fresh start for European science" (*Nature* 367, 592; 1994) prompts me to say that the European Environmental Research Organisation (EERO) already has a policy of sponsoring individuals rather than projects and has been doing so since it began in 1990. The output so far suggests that a focus on individuals is indeed cost-effective, and, compared with funding programmes and projects, is also less cumbersome.

Organizations such as the EERO aim to build on scientific opportunities likely eventually to produce useful results; they function best by funding the research leaders who identify these opportunities. The European Commission policy presumably responds essentially to political and socio-economic concerns and so can appropriately lead to the funding of customer-driven research programmes. Room should exist for both kinds of structure.

I read with interest Holland's comments about rationalizing PhD training and career development. A first step towards influencing education and training of graduates and undergraduates can be through involving in training courses the university staff responsible for teaching at those levels. The EERO already has a programme of such training courses in the environmental sciences. If one tries to develop guidelines as Holland suggests, they must somehow be compatible with the complex and heterogeneous course structures and curricula that exist across the continent of Europe. I am sure this must be feasible, but it will take time.

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Nuclear weapons

SIR — In Laurence Martin's review (*Nature* 367, 603-604; 1994) of the Pugwash book *A Nuclear-Weapon-Free World: Desirable? Feasible?* he notes the argument that the retention of nuclear weapons by some states is an assurance against their use on themselves. This does not sufficiently take into account the great unlikelihood that nations not now possessing nuclear weapons would forgo indefinitely the presumed advantages of having them.

The Non-Proliferation Treaty (NPT) will be considered for renewal in 1995, and dissatisfaction with the discriminatory nature of 'have' and 'have-not' nations has

been great among the 'have-nots'. In fact, this was why the NPT conference in 1990 could not agree on a final statement. The 1995 conference is thus in jeopardy, and we may face a breakdown in whatever restraint the NPT now exerts on 'have-not' countries that may be unwilling to continue their lessened political clout in comparison with the 'have' countries.

There is, of course, the unprovable assumption that the threatening postures of the United States and the former Soviet Union during the Cold War served to keep the world free of nuclear war. Clearly, the restraint and caution of the leaders of those countries played an important role. If some countries continue to assert their exclusive right to possess such weapons, however, the NPT will be at continuous risk. It is virtually certain that over time some 'have not' countries will not forgo the political and military status that the 'have' countries now possess. With the spread of such weapons to additional countries, and given the political instabilities which will surely continue, it would be foolhardy to depend on continued restraint by leaders in crises in an increased number of nuclear-weapon powers. This possibility plus the examples of Iraq, Libya, Iran and some African countries, apart from the non-declared possessors of nuclear weapons, should give pause to those who still support the possession of such arms; but apparently they remain impervious to the logical conclusion that should be drawn.

As the Pugwash book cogently argues, a nuclear-weapon-free world will be a difficult and long-term undertaking, but there is no alternative if we are to minimize the everlasting threat such weapons pose.

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Don't eat it

SIR — The debate on the use of bovine somatotropin (BST) to increase milk yield in dairy cows is not helped by misinformation on its apparent use in agricultural practice (*Nature* 367, 582; 1994). BST licensed for use in the United States is administered in a slow-release form by intramuscular injection not "by adding BST to feed". Feeding peptide hormones to cows would be an expensive form of protein supplementation in the diet with no effect on milk output; similar protein digestion would of course occur in humans consuming milk products containing the natural hormone.

Chris Seal

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How to hasten peace in Ireland

SIR — Recently, John Maddox briefly reviewed the origins and status of universities in Northern Ireland and the Irish Republic (*Nature* 368, 13; 1994).

I am an Englishman who spent 30 years at the Queen's University of Belfast and I believe that Maddox has committed the same error as many other Englishmen, especially politicians. Although the prospects for achieving a peaceful settlement in Northern Ireland appear unpromising in the short term, there is one guaranteed method of achieving agreement between the different factions of the population. Proposals for their future emanating from Englishmen who have never lived in Northern Ireland will evoke a united opposition.

Maddox suggests integration of the Irish universities. I believe that his well-meaning suggestion would fail for the reason I have given. He will be interested to learn, however, that biochemists from both parts of Ireland decided to form a group for intellectual exchange in 1966. The Biochemical Society gave its blessing to the scheme and the Irish Area Section of the society was formed. The society has funded an annual meeting, including a novel scheme for predoctoral students, at the various institutions. In addition, the National Committee for Biochemistry in the Republic has routinely had a representative from the Queen's University since then.

I know that other academic disciplines have been involved in similar academic collaborative exercises. These successes have resulted from the efforts of academics and students and not from the activities of politicians of either country.

My message then is not to force-feed the populace of either part of Ireland with a political diet that will evoke opposition rather than achieve the hoped-for peaceful settlement. Westminster and Dublin should devote less attention to sowing seeds of peace of their choosing and more to fostering any attractive seedlings that emerge from the grass roots. This is not a proposal for British disengagement but a plea for patience. In my view, it will take at least a generation for the population of Northern Ireland to recover from the trauma of the past 25 years. Altering the funding arrangements of universities, especially if that implies further academic economies, will do nothing to hasten the process.

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Change the rules for food additives

John Ashby

The Delaney clause, embodied in US legislation in 1958, prohibits the addition to food of any level of any carcinogen. This position cannot be sustained in the face of progress in understanding chemically induced cancer.

THE Delaney clause of the US Food Additives Amendment, 1958, states that "no [food] additive shall be deemed safe if it is found to induce cancer when ingested by man or animals...". According to its author, Representative James J. Delaney of New York, "carcinogens are subtle, stealthy, sinister saboteurs of life. They have no place in our food chain"¹.

In 1988, the US Environmental Protection Agency began granting tolerances under *de minimis* exceptions to the clause, permitting a risk of one case of cancer in 1 million people during 70 years, if within the context of a favourable risk/benefit assessment. But the US courts have decided that no such exceptions can be granted unless the Delaney clause is changed by Congress². The main scientific obstacles to change appear to be the assumptions implicit in the clause that all rodent carcinogens pose an equal hazard to humans, and that one molecule of any carcinogen, acting on a single cell, can induce tumours (see the Correspondence from T. Jukes on page 580).

Legislation to prevent addition of carcinogens to food seems to be an intrinsically good thing. But progress over the past 36 years in our understanding of chemically induced carcinogenesis has been so dramatic as to call into serious question the validity of the Delaney clause. Cicero stated that law is founded in nature, not in opinion — but the Delaney clause is founded in little more than opinion. Nonetheless, I believe, like Delaney, that there should be means to prevent the trivial addition to foods of carcinogens that are expected to cause cancer in humans.

In 1958, there were only a few known human carcinogens, such as benzidine, and a few known rodent carcinogens, such as benzantracene. The bioassay protocols used at the time recognized only clear, potent carcinogens. Within that context, legislation to prevent addition to food of such "sinister saboteurs of life" seemed to be justified. James and Elizabeth Miller then discovered that carcinogens, or their metabolites, are reactive to cellular macromolecules such as DNA, and Bruce Ames and subsequent workers established that they were also mutagenic (genotoxic) across phyla — carcinogens were mutagens with recognizable reactive centres within their chemical structures. Growing concern in the mid-1970s about the true prevalence of carcinogens in the

environment led the US National Cancer Institute and National Toxicology Program (NTP) to initiate a series of detailed cancer bioassays in rats and mice of chemicals of environmental importance.

To date, results from more than 300 chemicals have been published, only a third of which show no evidence of rodent carcinogenicity. About one half of the new carcinogens were of the classical genotoxic type — active in several tissues and/or in both species, and mutagenic. A further 60 were uniquely active at single sites of only one species, and then often in only one gender. In addition, the apparently benign chemical structures and the absence of mutagenicity for most of these 60 carcinogens are wholly inconsistent with a genotoxic mechanism of carcinogenic action³. These presumed non-genotoxic carcinogens are thought to increase the tumour incidence in rodents by physiological mechanisms associated with chemically induced cell division. The induction of renal tumours uniquely in male rats by the renal mitogen limonene (a constituent of limes and lemons) is the best studied non-genotoxic carcinogen (see ref. 4 for a review).

In the absence of the unifying hypothesis of DNA reactivity, any hazard these non-genotoxic carcinogens might pose to humans must be individually established, not assumed. Lemon juice cannot travel on the coat-tails of benzidine. Further, if the 100 or so non-carcinogens defined by the NTP were to be submitted to further bioassays in different species or strains of test animal, then many would probably show some non-genotoxic carcinogenic properties³. The term 'rodent carcinogen' has therefore drifted far away from the threat envisaged by Delaney.

The case of the flavouring agent and perfume additive benzyl acetate illustrates the point. In 1986, the NTP reported benzyl acetate to be carcinogenic when administered by oral gavage (intubation) in corn oil. Tumours were observed in the male rat pancreas and in the stomach and liver of both sexes of mice⁵. Benzyl acetate has a non-reactive chemical structure and is non-genotoxic⁶. The NTP expressed concern that the corn oil vehicle may itself have caused the pancreatic tumours, and that local irritation of the stomach induced by high local concentrations of the chemical might have caused the stomach tumours. The NTP therefore retested it in a feeding study, in which it was

found to be uniformly non-carcinogenic to rats and mice⁵.

What is one to make of such conflicts of data? First, unqualified use of the term 'rodent carcinogen' is an insecure basis for legislation. Second, physiological disturbances caused by maximum-tolerated doses of chemicals may lead to isolated instances of rodent carcinogenesis of dubious or no relevance to humans⁷. Third, a rational application of the Delaney clause to chemicals such as benzyl acetate is impossible; the overwhelming evidence is that benzyl acetate will be non-carcinogenic to humans, yet it remains a 'carcinogen' for legal purposes.

There are various ways in which a newly discovered rodent carcinogen can be classified as genotoxic or non-genotoxic^{8,9}. In the case of a probable genotoxic carcinogen, benefit/risk assessments can lead to *de minimis* exceptions in appropriate cases. In the case of a probable non-genotoxic rodent carcinogen, additional data, or data on analogues, can indicate if the carcinogenic effect is rodent-specific or if a hazard to humans exists. Many non-genotoxic carcinogens will be found on this basis to pose no risk to humans; for example, corn oil, lemon juice, oxazepam and benzyl acetate — but some might pose a hazard; for example, dioxin (TCDD).

Given that many rodent carcinogens are now known to be ubiquitous in foods and the environment, what is needed is a legal framework sensitive to advances in understanding of chemical carcinogenicity. This will allow the rational use of all relevant general information on chemical carcinogenicity, coupled to that for the particular agent under study, leading to an estimate of carcinogenic hazard (or lack of it) to humans. Such an integrated process should be the key to effective human health protection, not only for food additives, but for pesticide residues, therapeutic drugs and environmental chemicals. □

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1. Jukes, T. H. *Prev. Med.* **2**, 133–139 (1972).
2. *Nature* **363**, 287 (1993).
3. Ashby, J. & Purchase, I. F. H. *Mutagenesis* **8**, 489–493 (1993).
4. Swenberg, J. A. *Environ. Hlth Perspect.* **101** Suppl. 6, 39–44; Suppl. 5 (1993).
5. NTP Technical Reports, 250 (1986); 431 (1993).
6. Ashby, J. *Mutat. Res.* **306**, 107–109 (1994).
7. Ames, B. N. & Gold, L. S. *Science* **249**, 970–971 (1990).
8. Hildebrand, B. *Mutat. Res.* **248**, 211–374 (1991).
9. Ashby, J. & Morrod, R. S. *Nature* **352**, 185–186 (1991).

Rolling back the frontiers in the brain

A global scientific programme using a sweeping range of techniques to tackle some of the most daunting problems in biology ought to be impossible. But it works.

IN one of the first talks at the conference on "Information Storage, Transfer and Retrieval" organized by the Human Frontier Science Program (HFSP) in Hakone, Japan, on 5–8 April, Lubert Stryer (Stanford Medical School) told of a conversation in 1969 with Henri Peyre, then professor of French at Yale. Unimpressed by Stryer's account of how he intended to determine the molecular basis of vertebrate vision, Peyre remarked that the truly interesting question was the molecular basis of remorse.

That goal is not yet within reach, but as a statement of the HFSP's bold ambitions, it could hardly be bettered. The programme's review committee aims to select the best proposals for studies of the human brain from Japan, Europe and North America, using techniques ranging from molecular biology to computer modelling. By no means all of the work described at this meeting was supported by the HFSP, but enough of it was to suggest the HFSP is in good shape.

Not all of the work described concerned the nervous system. The helices formed by linear polypyridines (Jean-Marie Lehn, Université Louis Pasteur, Strasbourg, and Collège de France, Paris), the unexpected interdependence of elements in the *c-fos* promoter (Tom Curran, Roche Institute of Molecular Biology, New Jersey), and the tumour-suppressor activity of the interferon-induced transcription factor IRF1 (Tadatsugu Taniguchi, Osaka University) clearly have implications for all cells.

But, in general, the neural theme was dominant. Discussion of neural lineage commitment focused on *Drosophila* bristle-formation; both the transcription factor JkRBP (Tasuku Honjo, Kyoto University) and the signal-receptor pair *Delta* and *Notch* (Patricia Simpson, Institut de Chimie Biologique, Strasbourg) are important. Two molecules directing axonal targeting were also featured (Corey Goodman, HHMI, University of California, Berkeley, and Friedrich Bonhoeffer, Max Planck Institut, Tübingen); although the first, at least, appears to be a homophilic cell-adhesion protein, like the second it can also repel non-expressing neurons.

Nor was neuronal signalling neglected. Jean-Pierre Changeux (Institut Pasteur, Paris) described mutations in the amino acids lining the pore of the nicotinic acetylcholine receptor that convert it into an anion channel. Signal termination, too, was scrutinized (Stryer); excited photorhodopsin in vertebrate rods stays active until falling intracellular calcium levels detach two calcium ions bound by the protein recoverin,

freeing recoverin from the membrane by inducing retraction of its phospholipid anchor.

Such phenomena can directly affect animal behaviour. Conditioning of the startle response in fish depends on a new-found plasticity in gap junctions and inhibitory synapses, while some inhibitory neurons become active only on conditioning (Henri Korn, Institut Pasteur, Paris). Just as striking is the modulation of GABA transmission in the accessory olfactory bulb by type 2 metabotropic glutamate receptors (Shigetada Nakanishi, Kyoto University); the resulting olfactory memories appear necessary, *inter alia*, for sexual fidelity in female rats.

But the molecular basis of most processing is less clear. Habit formation and visual recall are mediated by anatomically and pharmacologically distinct systems in monkeys (Mortimer Mishkin, NIMH, Bethesda), but study of the synapses involved lies ahead. Markus Raichle's studies of human habit formation using ^{15}O positron emission tomography suggest a related distinction (University of Washington, St Louis).

A different specialization is evident in the prefrontal cortex (Patricia Goldman-Rakic, Yale University School of Medicine, and Michael Petrides, Montreal Neurological Institute): the position, features and list order of visual objects are all handled by separate regions. Even more remarkably, a nearby region on the left side of this area appears to mediate discrimination of events closely spaced in time (Paula Tallal, Rutgers University). Ninety per cent of dyslexic patients lack this ability, and cannot distinguish syllables differing only in their first 40 milliseconds, but the problem affects other senses as well. The lateralization of speech, rather than being uniquely human, may therefore represent an adaptation of previously existing neuronal circuitry, an idea supported by the recent demonstration that rats share the 'right-ear advantage' previously thought to be confined to humans.

But fine temporal discrimination is involved in other phenomena as well. The inositol triphosphate and ryanodine receptors may in fact act as 'coincidence detectors', releasing calcium only when triggered both by cytoplasmic calcium and their respective second messengers (Michael Berridge, University of Cambridge). And the synchronized waves of activity that sweep the ganglion cells of each ferret retina before they receive visual input are vital if each eye is to be correctly connected to separate layers in the lateral geniculate nucleus (Carla Schatz,

University of California, Berkeley).

The features of a single object may even be grouped by synchronizing the firing of all neurons involved, allowing overlapping percepts to be rapidly distinguished (Wolf Singer, Max Planck Institute for Brain Research, Frankfurt). Multielectrode recordings of amblyopic strabismic monkeys show that cortical neurons driven by the disadvantaged eye indeed encode object features normally but fire asynchronously in response to stimuli the monkey cannot see.

Elsewhere, however, information is stored differently. Neurons in the inferior temporal cortex of monkeys trained to associate paired abstract designs show highly selective responses to pair members (Yasushi Miyashita, University of Tokyo). Similarly, simultaneous recording of as many as 148 neurons in the hippocampus of an active rat shows that most cells monitored respond selectively to particular locations (Bruce McNaughton, University of Arizona, Tucson). An intriguing aspect of this technical *tour de force* is that cells whose firing was correlated during exploration were again co-activated during slow-wave sleep, suggesting that the hippocampus 'downloads' memories to the cortex while it is 'off-line'.

But it is in modelling brain function on the basis of such information that the HFSP's breadth is most clearly beneficial. McNaughton's data, for instance, have been used to build a model of hippocampal function in which a partial cue can elicit information stored in the cortex to reinstate the memory (Edmund Rolls, University of Oxford). Even more impressive are cases in which the model not only correctly predicts neuronal responses to a given stimulus, but can be shown to improve robot arm performance (Mitsuo Kawato, ATR Human Information Processing Research Laboratories, Kyoto) or to respond in a life-like way to complex visual illusions (Terrence Sejnowski, Salk Institute, San Diego).

Despite such spectacular advances, it will be a long time before the stupendous complexity of most neural systems is understood. But there can surely be few things more interesting to mankind than our brains, and, the HFSP has clearly hit on a winning formula for their study. It is therefore particularly sad that more countries have not emulated Japan's example and given the programme the financial support it deserves. Other member nations should be overcome by remorse, whether they understand its molecular nature or not, and reach for their cheque books.

Nicholas Short

Distantly detecting deuterium

John N. Bahcall

A NEW era of astronomy is heralded by the article¹ on page 599 of this issue. There, Songaila *et al.* present two results that could be of fundamental significance to cosmology: the first tentative detection of deuterium outside our Galaxy, at a large redshift ($z = 3.3$), and a meaningful upper limit on the temperature of the cosmic background radiation at a different large redshift ($z = 2.9$).

The first result extends the tests of the Big Bang picture for the synthesis of light elements in the early Universe to deuterium, which is an exquisitely sensitive measure of the total amount of baryonic matter (the protons and neutrons of 'ordinary stuff') in the Universe. It is either a tentative measurement or a definitive upper limit of the abundance of deuterium, as explained below. Previously, among the light elements, only the abundances of ordinary hydrogen and of helium had been measured outside our own Galaxy. The second result tests the essence of the idea that the cosmic microwave background radiation is indeed what it seems to be, a universal remnant of the Big Bang that obeys the laws of general relativistic cosmology.

Astronomers are bumping into each other, dancing in the dark corridors of their observatories. Why are they so happy? They are thrilled because the techniques used by Songaila and her colleagues show that the first of the two giant 10-m Keck telescopes on Mauna Kea, Hawaii, is a great success, and that some of the questions that astronomers have sought to answer for decades may be solved in a night's observations with these new eyes. The authors recount how they and other astronomers have previously struggled unsuccessfully, using more modest telescopes (4-m and 5-m class), to measure the deuterium abundance and the temperature of the cosmic background radiation at large redshifts.

The key to the present success is the marvellous operation of the Keck telescope, which captures light from distant objects with an efficiency and a sensitivity that makes easy what was until recently impossible. For the sensitive cosmological tests that Songaila *et al.* attempt, it is necessary to observe faint objects far away with a high ratio of signal to noise. Obviously, in this case, big is better.

Songaila *et al.* analyse a nearly primordial cloud of absorbing hydrogen gas that is illuminated by the light of a distant

quasar, first examined in detail by Chaffee and collaborators² using the Multiple Mirror Telescope of the University of Arizona. This cloud has many characteristics that permit an accurate determination of the ratio of deuterium to ordinary hydrogen, D/H. The ratio that Songaila *et al.* measure, $D/H = 2.5 \times 10^{-4}$, is much higher than the ratio determined — after significant corrections for local effects — in the nearby interstellar medium of our own Galaxy³. Moreover, the high deuter-

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Keck — providing a clearer view of the Universe.

ium abundance indicated by the new observations has the effect, within the theory of nucleosynthesis of light elements, of reducing the calculated total abundance of baryons to about the value that is estimated from observations of all of the luminous matter in the Universe⁴. This inference, if correct, would imply that the massive haloes of unseen material that are believed to surround ordinary galaxies must be made of nonbaryonic matter, a result of enormous significance for physics and astronomy.

In one of those remarkable coincidences that are surprisingly common in the history of science, R. Carswell⁵ and an international collaboration of scientists have obtained very similar results on the deuterium-to-hydrogen ratio in exactly the same hydrogen cloud. The two analyses are independent and use different data obtained with different telescopes and spectrographs (Carswell's team used the 4-m Kitt Peak National Observatory telescope in Arizona), so the similarity of their answers increases confidence in the results.

A word of caution is appropriate. Songaila *et al.* estimate that there is a three per cent chance that the absorption line that they think is caused by deuterium might instead be caused by absorption from a different hydrogen cloud, one that just happens to be placed so as to be confused with deuterium from the cloud they are studying. Carswell *et al.* make a similar cautionary statement. Not to worry: Keck can make many such observations. We should know within a year or two whether this first cosmological observation of the deuterium-to-hydrogen ratio was confounded by bad luck.

According to the basic ideas of relativistic cosmology, the temperature of the cosmic background radiation must scale in proportion to $(1 + z)$, where z is the redshift at which the radiation is measured. It was suggested a quarter of a century ago⁶ that this predicted increase of temperature with redshift could be tested by observing the populations of excited fine-structure lines in the absorption spectra of distant quasars. Earlier attempts to observe this effect were limited by the signal-to-noise ratios obtainable with smaller telescopes^{7,8}. Songaila and co-workers, with the first Keck observations searching for excited fine-structure lines, find a 2σ upper limit of 13.5 K for the temperature of the cosmic microwave background in a cloud that contains⁹ singly ionized carbon at $z = 2.9$. This upper limit lies very close to the temperature, 10.7 K, expected on the basis of relativistic cosmology. Songaila and her colleagues appear confident that definitive measurements at large redshifts will be possible with Keck.

These two new observational tests of relativistic cosmology will be among the many important questions that can be investigated once the new generation of large optical telescopes becomes more readily available to astronomers. Basic ideas of homogeneity and isotropy that are inherent in the standard Big Bang hypothesis predict that the primordial deuterium-to-hydrogen abundance ratios measured in different directions and at different cosmological distances will all be

Roger Ressmeyer, Starlight/Science Photo Library

1. Songaila, A., Cowie, L. L., Hogan, C. J. & Rugers, M. *Nature* **368**, 599–604 (1994).
2. Chaffee, F. H., Foltz, C. B., Roser, H.-J., Weymann, R. J. & Latham, D. W. *Astrophys. J.* **292**, 362 (1985).
3. Linsky, J. L. *et al.* *Astrophys. J.* **402**, 694–709 (1993).
4. Walker, T. P., Steigman, G., Schramm, D. N., Olive, K. A. & Kang, H. S. *Astrophys. J.* **376**, 51–69 (1991).
5. Carswell, R. F. *et al.* *Mon. Not. R. astr. Soc.* (in the press).
6. Bahcall, J. N. & Wolf, R. A. *Astrophys. J.* **152**, 701–729 (1968).
7. Meyer, D. M., Black, J. H., Chaffee, F. H., Foltz, C. & York, D. G. *Astrophys. J.* **308**, L37–L41 (1986).
8. Bahcall, J. N., Joss, P. C. & Lynds, R. *Astrophys. J.* **182**, L95–L98 (1973).
9. Sargent, W. L. W., Steidel, C. C. & Boksenberg, A. *Astrophys. J. Suppl.* **69**, 703–761 (1989).

the same. Similarly, if the ideas of relativistic cosmology are correct, the temperature of the cosmic microwave background must be the same in all directions at the same distance and must scale as expected with redshift. All of this will be tested experimentally in future.

Astronomers and physicists are confident of the outcome, nearly certain that the fundamental concepts of cosmology and of Big Bang nucleosynthesis will once

again be found to be consistent with observations. But perhaps present-day scientists are slightly less confident than their intellectual predecessors, Michelson and Morley, who set out to measure the velocity of the Earth with respect to the aether. □

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SOLAR SYSTEM

Heliosphere in a bottle

W. S. Kurth

WITH Pioneers 10 and 11 and Voyagers 1 and 2 now forming a veritable fleet of heliospheric probes, interest in the outermost reaches of the Sun's influence is mounting. When can we expect them to reach the regions where the solar wind's influence gives way to that of the local interstellar medium, and what will be seen there? These questions have led S. Minami to make the first laboratory simulations of the interaction¹.

First, the context of the problem. The solar wind, a stream of ionized solar gas with its associated magnetic field, flows radially outwards at supersonic velocities throughout the Solar System. Initially, it should expand more or less spherically, but must eventually collide with the local interstellar medium (ISM) through which the Solar System is moving. It is believed that the solar wind becomes subsonic at the termination shock, well inside the heliopause, yet beyond the current positions of the Voyager and Pioneer spacecraft. The shape of the heliopause — the boundary of the region of space dominated by the Sun's plasma — will depend on the relative velocities and, as a further complicating factor, on the interstellar magnetic field in our locality.

Some evidence of the interaction between the solar wind and ISM is already emerging, including low-frequency radio emissions possibly from the heliopause at distances of 116–177 astronomical units (1 AU is the distance from Earth to Sun)², and evidence for the termination shock in Voyager and Pioneer observations of the hydrogen Lyman- α lines³.

But even if one or more of the probes directly samples the interface, models will be important in making sense of the observations and fitting them into a global picture. There have been numerous analytical and numerical simulations of this interaction, but all have suffered from the simplifying assumptions required to make the problem tractable. To be sure, Minami's laboratory simulations have their own limitations, but it is good to see that laboratory plasmas and theory can be

used in complementary ways to fill in the gaps in our knowledge of the heliosphere.

In Minami's experiment, a barium plasma (representing the solar wind) expands into a flowing argon plasma with a variable magnetic field (representing the local ISM). Both flows are supersonic. The barium plasma is luminous, so a high-speed camera can take global images of the simulated heliosphere.

The primary purpose of the initial work is to investigate how sub- or super-Alfvénic flow of the local ISM alters the shape of the heliopause. The Alfvén velocity refers to the velocity of propagation of low-frequency magnetohydrodynamic waves in which the fluid provides the inertia and the magnetic field the restoring force. This velocity is proportional to the background magnetic field. The Alfvén Mach number is the ratio of the flow velocity to the Alfvén velocity; hence, Alfvén Mach numbers greater than 1 imply super-Alfvénic flow.

The shapes obtained for sub- and super-Alfvénic flow are rather different. In the two sub-Alfvénic cases that Minami looks at, the 'heliopause' develops a sharply pointed nose oriented into the ISM flow and an open tail streaming away from it (the exact direction of the tail depends on the angle of the interstellar field, and there is strong axial asymmetry if this is not parallel to the flow direction). The super-Alfvénic case (at low magnetic field) produces a parabolic shape to the nose, similar to that seen in many of the theoretical models. As we do not know all of the essential parameters of the interstellar wind, we cannot assume that the agreement implies that the flow is super-Alfvénic; rather, it suggests that the two independent methods of modelling the interface provide the same qualitative results and, therefore, at least partially validates the modelling approaches.

Minami estimates that the simulation implies distances to the heliopause in the range 200–400 AU, but he cautions that the scaling of distances in the experiment is more qualitative than quantitative.

This is evidently only the first in a series of experiments which can be done in the laboratory, and the work promises much more in the way of parametric studies of the interaction between solar wind and ISM. Its great advantage is the relative ease of varying important parameters such as the strength and orientation of the interstellar magnetic field to obtain even a qualitative understanding of their effect on the characteristics of the interaction. Analytical and numerical modelling has been limited in the ability to include such effects, particularly asymmetries.

A good example of numerical modelling appeared about the same time as Minami's laboratory work. In this model, Steinolfson and co-workers⁴ use a gas dynamic model for the interaction without including the interstellar magnetic field. (Neither model includes the magnetic field of the solar wind.) In apparent agreement with Minami's super-Alfvénic result, the numerical model suggests a parabolically shaped nose, but the comparison cannot be taken further as the particular problem set up by Steinolfson *et al.* does not allow axial asymmetries. Again, the authors promise future work with additional effects included.

Modelling the heliosphere promises to continue with increasing intensity and fidelity as the deep-space probes approach and overtake the termination shock. The list of effects that various researchers urge should be incorporated is impressive: it includes the question of whether the shocked solar wind is compressible, the interplanetary magnetic field, resonant charge-exchange effects between interstellar hydrogen and the plasma, pickup of interstellar hydrogen in the solar wind, cosmic rays and the anomalous component of the cosmic-ray spectrum, the solar cycle, interstellar magnetic field strength and orientation, and a wide range of interactions at the heliopause which may or may not be analogous to effects at the Earth's magnetopause such as diffusion and reconnection. (See ref. 5 for a summary — although not an exhaustive review — of some of these modelling efforts.)

Given the sheer number of possible effects, Minami's type of experiment should be welcome to heliospheric physicists as a chance to compare the theoretical models to a real plasma system, as limited in its similarity to the actual heliosphere as it might be. □

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1. Minami, S. *Geophys. Res. Lett.* **21**, 81–84 (1994).
2. Gurnett, D. A. *et al.* *Science* **262**, 199–203 (1993).
3. Hall, D. T. *et al.* *J. geophys. Res.* **98**, 15185–15192 (1993).
4. Steinolfson, R. S., Pizzo, V. J. & Holzer, T. *Geophys. Res. Lett.* **21**, 245–248 (1994).
5. Ratkiewicz, R. *Adv. Space Res.* **13**, 179–184 (1993).

Bonanza at Shanghuang

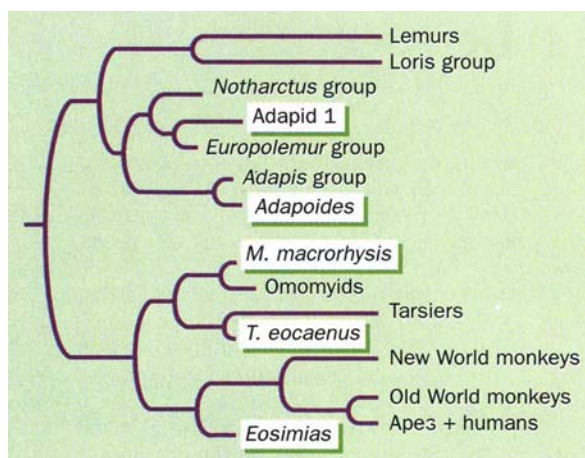
R. D. Martin

THE rate of discovery of early fossil primates has shown a pleasing surge in the 1990s. These finds¹⁻⁶ — in North America, Europe and north Africa — have enriched our understanding both of the early radiation of prosimians, including the lemur-like adapids and tarsier-like omomyids, and of the origins of 'higher' simian primates (monkeys and apes). But even taken together, they are overshadowed by the contents of a new primate fossil site, described by Beard *et al.* on page 604 of this issue⁷, at Shanghuang in southeastern China. At one fell swoop, this site has yielded five new types of early primate, each representing a distinctive lineage in the early adaptive radiation of the group (see figure). This diversity of early primates in a single place is greater than for the rest of Asia put together, and indeed exceeds that found at better-documented sites in Europe and North America.

The Shanghuang site is unusual for primates in that the rich fossil deposits exist as accumulated fissure-fillings in Triassic rock. Their absolute age has not yet been determined, but comparisons based on various specimens from the diverse mammalian fauna permit relative dating to the middle Eocene (about 45 million years ago). One feature of the fissure-fillings is that the sample of early mammals seems to be derived from a forested environment, which would explain why primates are so well represented. There is, however, a disadvantage in that material accumulated in fissures tends to be very fragmentary, in the form of isolated teeth and pieces of jaw; in contrast to sedimentary deposits, there is probably a bias towards relatively small-bodied mammals. All of the Shanghuang primates are quite small and we should therefore be wary of concluding either that all middle Eocene primates of the region were small-bodied or that the deposits contain samples of all of the species that lived there.

The discovery of adapids and omomyids in Eocene deposits is not unexpected. Nonetheless the new specimens provide a great deal to think about. For instance the Shanghuang adapids seem to represent two distinct lineages. Upper molar teeth of the first (unnamed) type resemble those of *Europolemur* from European deposits of similar age. There could be an affinity to the unusual late Eocene adapid *Mahgarita* of North America, and Beard

et al. suggest that the latter genus may therefore have originated in Asia. But the molar teeth of the unnamed Shanghuang adapid are relatively primitive (for instance the upper molars lack a fourth cusp, the hypocone), so the affinity both to *Europolemur* and to *Mahgarita* may be tenuous. The teeth of the second adapid,



Outline phylogenetic tree of primates showing the six major groups of living species (lemurs, lorises, tarsiers, New World monkeys, Old World monkeys, apes + humans), and one interpretation of their relationships to certain early fossil groups. (Others are possible, but do not greatly affect the arguments presented here.) The five new fossil primates from Shanghuang (boxed) include early representatives of several different lineages: two different adapids (the unnamed adapid 1 resembling *Europolemur* and the second, *Adapoides troglodytes*, showing similarity to European adapids of the *Adapis* group); an omomyid (*Macrotarsius macrorhysis*); a species allocated to the modern genus *Tarsius* (*T. eocaenus*); and the inferred basal simian *Eosimias sinensis*.

Adapoides troglodytes, are similar in size to those of the European *Microadapis* and there is a general resemblance in dental features to genera belonging to the tribe Adapina. This group contains a number of specialized forms that appear abruptly late in the Eocene in Europe, and so we now have the possibility that the Adapina originated in Asia and later migrated into Europe.

Isolated lower cheek teeth of the omomyid discovered at Shanghuang closely resemble those of the distinctive North American *Macrotarsius* from the middle Eocene to the early Oligocene. The new Chinese species has accordingly been named *M. macrorhysis*. As well as documenting a further primate lineage in the region, this indicates the existence of a direct link between Asian and American omomyids during the Eocene.

Perhaps the most spectacular of the Shanghuang finds consists of isolated cheek teeth (lower molars and an upper premolar) that are practically indistinguishable from those of modern tarsiers.

The modern genus *Tarsius* contains five closely related species confined to islands in southeast Asia, and until eight years ago there was no known fossil record. The age of the genus was then pushed back to the early Miocene by the discovery in Thailand of dental remains allocated to the species *Tarsius thailandica*⁸. The Shanghuang specimens have also been allocated to *Tarsius* (species *T. eocaenus*), which represents a dramatic increase in the antiquity of that genus by almost 30 million years. Occurrence of a modern primate genus as far back as the Miocene has a precedent in the recognition of *Aotus dindensis* from Colombia as a direct relative of modern owl monkeys⁹, but extension of a modern genus back as far as the Eocene is unique among primates.

This raises the intriguing possibility that tarsiers not very different from their modern relatives may have inhabited Chinese forests 45 million years ago, hence justifying the tag 'living fossil' for *Tarsius*. It is noteworthy that the teeth of *T. eocaenus* are smaller than those of the smallest living species, *T. pumilus*, which might indicate that the first tarsiers were even smaller than their modern relatives. Another implication of the Shanghuang fossil tarsiers is that the Shanghuang fauna inhabited rainforest, because modern tarsiers live exclusively in rainforests.

As yet, though, we have only isolated teeth of *T. eocaenus*. There is a potential problem here in that *Tarsius* seems to have the most primitive molar teeth of

modern primates — for example, the upper molars are simple three-cusped teeth, whereas the lower ones retain a well-developed paraconid cusp, such that the original triangle is preserved there, too. The similarity between *T. eocaenus* and modern tarsiers is thus to some extent based on primitive retention. Nonetheless the similarity is so close in fine details that a direct link between the Eocene form and modern *Tarsius* seems likely. In any case, the awkward possibility that the teeth of modern tarsiers had been secondarily simplified in the course of evolution is now ruled out¹⁰. The existence of virtually identical molar teeth 45 million years ago indicates fairly clearly that the molar morphology of modern tarsiers is, indeed, primitive. But it remains to be seen whether *T. eocaenus* already had the distinguishing features of modern tarsiers, such as extremely large eyes and fusion of the fibula with the tibia.

The fifth new primate to emerge from Shanghuang, *Eosimias*, is also of great

significance, as it has been identified as a basal simian. *Eosimias* is known only from partial mandibles, but it is possible to reconstruct the lower dental formula as 2.1.3.3., corresponding to the expectation for an ancestral simian. Although *Eosimias* has several primitive features, there is a contrast to adapids, omomyids and modern prosimians in that the jaw symphysis was upright (as is typical of simians) rather than inclined. Another special feature is that the roots of the central and posterior lower premolars were obliquely arranged, reflecting compression of the anterior part of the cheek tooth row. Further, the anterior lower premolar was single-rooted. It is on the basis of these and other features that *Eosimias* has been interpreted as a relatively primitive form linked to the origin of simians.

Clearly, more attention should now be directed at three other late Eocene genera from Asia: *Amphipithecus* and *Pondaungia* from Burma and *Hoanghoni* from China. These enigmatic forms, known only from a few fragmentary specimens, have been variously interpreted as early simians or as adapids. Given that Beard *et al.* have identified several similarities between *Eosimias* and *Amphipithecus*, these other Asiatic forms now need careful re-examination. The result may be a complete reappraisal of simian evolution in Asia.

The Chinese finds also add a further dimension to the debate about possible adapid origins of simians. There are undoubted dental resemblances between at least some adapids and simians, but it is unclear whether these similarities reflect a phylogenetic relationship or are merely the result of convergent evolution¹¹. If *Eosimias* is indeed a basal simian, any direct phylogenetic relationship between adapids and simians is unlikely for three main reasons. First, Beard *et al.* note that there are specifically simian features in the dentition of *Eosimias* (and, for example, in *Amphipithecus*) which are generally lacking in adapids. Second, *Eosimias* has retained a number of primitive features, absent in later adapids, that are supposedly linked to simians; so evolution of *Eosimias* from such adapids would require evolutionary reversal. Third, if *Eosimias* is indeed an early simian, an adapid origin of simians seems implausible, as all of the adapids supposedly documenting a link to simians are geologically younger.

Beard and colleagues' discoveries additionally introduce a fresh perspective to possible times of origin of individual lineages in the primate tree. I have argued¹¹ that there has been a tendency to underestimate times of origin of primate lineages, and the Shanghuang material strengthens the case. It is, for instance, now widely accepted that tarsiers and simians (the haplorhine primates) shared a specific common ancestry. If definite

relatives of modern tarsiers were already present in the middle Eocene, then the date of origin of the haplorhines must have been much earlier.

The question of the relationship between omomyids and tarsiers is still open to debate, but some authors regard these taxa as sister-groups. If that is the case, there must have been a common ancestor for omomyids and tarsiers between the initial divergence of the haplorhines and the origin of tarsiers. Indeed, it has been suggested that tarsiers may be more closely related to the North American omomyid *Shoshonius* than to other omomyids¹ and this would require considerable diversification of omomyids prior to the emergence of tarsiers. Such considerations, added to the presence of the possible basal simian *Eosimias* in the middle Eocene, imply that haplorhine primates underwent a major radiation before the Eocene. If we then turn to the adapids, which probably diverged prior to the origin of the haplorhines and are also present at Shanghuang, the likelihood is that the primates of modern aspect originated in the Cretaceous.

Finally, the site at Shanghuang has, at a stroke, revolutionized our appreciation of the involvement of Asia in the early evolution of primates. And this is by no

means the end of the story. Egypt and Algeria apart, the African fossil record of Eocene primates is still very poorly known, and we can expect further surprises if Eocene or earlier sites are opened up south of the Sahara. It may be only a matter of time before early relatives of lemurs and lorises, possessing a fully developed tooth-comb (a diagnostic feature of these primates), are found in Eocene or earlier deposits in Africa. □

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1. Beard, K. C., Krishtalka, L. & Stucky, R. K. *Nature* **349**, 64–67 (1991).
2. Moya Solà, S. & Köhler, M. *Folia Primatol.* **60**, 158–163 (1993).
3. Moya Solà, S. & Köhler, M. *Nature* **365**, 543–545 (1993).
4. Simons, E. L. *Proc. natn. Acad. Sci. U.S.A.* **89**, 10743–10747 (1992).
5. Simons, E. L. *Science* **247**, 1567–1569 (1990).
6. Godinot, M. & Mahboubi, M. *Nature* **357**, 324–326 (1992).
7. Beard, K. C., Qi, T., Dawson, M. R., Wang, B. & Li, C. *Nature* **368**, 604–609 (1994).
8. Ginsburg, L. & Mein, P. C. *R. Acad. Sci., Paris, sér. II* **304**, 1213–1215 (1986).
9. Setoguchi, T. & Rosenberger, A. L. *Nature* **326**, 692–694 (1987).
10. Martin, R. D. *Primate Origins and Evolution: A Phylogenetic Reconstruction* (Chapman & Hall, London/Princeton University Press, New Jersey, 1990).
11. Martin, R. D. *Nature* **363**, 223–234 (1993).

VERTEBRATE DEVELOPMENT

On making a skeleton

Cheryl I Tickle

THE report by Storm *et al.* on page 639 of this issue¹ will be of considerable interest to those studying developmental biology in general, and limb development in particular, as well as to those involved in connective tissue biology. The authors have identified the gene affected in a mouse mutant, *brachypodism*, which suffers from defective skeletal development. They find that the gene — *Gdf5* — encodes a protein that, with two others, constitutes a new subgroup related to the so-called bone morphogenetic proteins.

The overall object of this area of research is to identify, at a molecular level, the factors involved in moulding the morphology of the skeleton. The basic shape of individual bones is first laid down in cartilage. So what determines the form of these individual cartilage rudiments, and their arrangement to give structures such as fingers and toes?

Interpretation of positional cues in the early limb bud leads to skeletal pattern². The limb develops from a bud of undifferentiated mesenchyme cells covered by an epithelium. It is the mesenchyme that differentiates into connective tissues such as those of the skeleton, both epithelium–mesenchyme and mesenchyme–

mesenchyme interactions being required for the process. Among the signalling agents concerned are retinoids (which can mediate substantial pattern changes when applied to chick wing buds but whose role in the normal limb is more controversial) and the vertebrate *hedgehog* protein³. The *hedgehog* signal seems to be involved in determining skeletal element identity across the limb and thus is instrumental in determining whether, for example, a finger will be a thumb or a little finger. Fibroblast growth factor(s)⁴ can provide the signal that makes the limb bud elongate and allows the sequential laying down of 'bones' along the length of the limb ending with fingers or toes at the tip.

The upshot of these signals, it is thought, is that patterns of Hox gene expression are established that give cells a positional address. It is then the interpretation of this positional information that leads to the appropriate development of particular bones. Conceptually, however, there is still a yawning gap between, say, a pattern of Hox gene expression and the cellular processes that are used to make an individual part of the skeleton.

A look at the early stages of element formation within the limb may help to

start bridging this gap. Skeletal elements first begin to be faintly discernible as cells in particular regions become more densely packed. Once cells are in this condensed state, they change the mix of matrix molecules they synthesize and later begin to produce large amounts of cartilage-specific molecules such as collagen type II. As matrix accumulates, the founding group of cells enlarges and cells become flattened around the periphery to give a sheath known as the perichondrium.

A series of signals seems to direct cell differentiation in the early rudiments of the skeleton, and Hogan and co-workers⁵ proposed a scheme in which members of the transforming growth factor- β (TGF- β) superfamily act sequentially in skeletal development. From expression pattern data of gene transcripts in early mouse limb buds, they suggested that the bone morphogenetic protein, BMP2, is the first signal and that, later, TGF- β s come into play. Bone morphogenetic proteins are the active ingredients of bone extracts that can induce bone in non-bony sites in adult animals⁶. The extraordinary feature of this induction is that cartilage is formed first and later ossifies, just as in embryonic development. Seven BMPs have been isolated from such extracts, most of which can induce bone.

What Storm *et al.* have now done is to identify three molecules — GDF (for growth/differentiation factors) 4, 5 and 6 — that are closely related to BMPs. Moreover, they provide direct evidence that these proteins are directly involved in limb skeletal development and could be a link between pattern formation and skeletal morphogenesis. In this context, it is intriguing that the *Drosophila decapentaplegic* gene, which is the direct homologue of *Bmp2* and *Bmp4*, acts downstream of *hedgehog*⁷. In vertebrate limb development, too, BMP2 may act in the same pathway as *hedgehog*⁸.

The *brachypodism* mutation is the second to be identified in a gene coding for a BMP-like protein. The first was *short ear*, a mutation of BMP5 (ref. 9), which not only results in vertically challenged ears but also skeletal abnormalities. These mostly affect axial parts of the skeleton, such as the sternum, a vertebra and the ribs; unlike *brachypodism*, the elements of the limb skeleton are, on the whole, normal in shape and size.

This raises the tantalizing possibility that different members of the BMP protein family could operate in different parts of the skeleton, putting flesh on an idea proposed some time ago. This was that cartilage cells are non-equivalent¹⁰; that is, even though they all differentiate as cartilage, they may have different positional addresses. This non-equivalence could determine cell behaviour and could, for example, control subsequent growth and shape of skeletal elements. Alternatively,

cartilage cells may be all the same, and it is the cells that surround the developing rudiment, such as those of the perichondrium, that carry positional information and control early molecular events and cell behaviour. This would make development of the skeleton similar to that of the musculature.

The *brachypodism* mutants have short limbs and tiny paws, and reduced numbers of bones in the digits. How could lack of expression of GDF5 protein lead to these changes? In cell culture, BMPs enhance cartilage differentiation of limb mesenchyme cells. In *brachypodism*, the early rudiments appear to be affected and Storm *et al.* show that *Gdf5* is expressed in early cell condensations and the perichondrium of the limb skeleton. So it looks as though this protein is acting very much in the way suggested by Hogan and co-workers⁵ for other BMPs, as an early promoter of skeletal development but specifically in the limb. This could mean that the number of founder cells for each limb element is reduced in the mutant and may account for the marked shortening of the limb skeleton. The abnormalities in the number of 'bones' making up individual digits is less easy to explain. There

are also fewer segments in some digits of a Hox-gene mouse 'knockout'¹¹. The Hox genes may be involved in growth, and it could be that it is insufficient growth that has led to the digit defects.

In all, it is encouraging that the molecules that control cell differentiation and skeletal form are beginning to be identified. But there is a long way to go in understanding how cells interpret positional information in the embryo and then work together to make each individual bone. □

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1. Storm, E. E. *et al.* *Nature* **368**, 639–643 (1994).
2. Wolpert, L. *Development* **107** Suppl., 3–12 (1989).
3. Riddle, R. D. *et al.* *Cell* **75**, 1401–1416 (1993).
4. Niswander, L. *et al.* *Cell* **75**, 579–587 (1993).
5. Lyons, K. M. *et al.* *Genes Dev.* **3**, 1657–1668 (1989).
6. Wosney, J. M. *Prog. Growth Factor Res.* **1**, 267–280 (1990).
7. Basler, K. & Struhl, G. *Nature* **368**, 208–214 (1994).
8. Francis, P. H. *et al.* *Development* **120**, 209–218 (1994).
9. Kingsley, D. M. *et al.* *Cell* **71**, 399–410 (1992).
10. Lewis, J. H. & Wolpert, L. *J. theor. Biol.* **62**, 479–490 (1976).
11. Dollé, P. *et al.* *Cell* **75**, 431–441 (1993).

ECOLOGY

The yucca expediency

Peter D. Moore

WITH the possible exception of fig wasps, there can be few insect pollinators that have a higher scientific profile than the yucca moth. This loyal lepidopteran, *Tegeticula yuccasella*, is the sole pollinator of many species of the Agavaceae genus *Yucca* and, in return, it lays its eggs in the pollinated yucca fruit where they hatch and produce the next moth generation. The association is often quoted as a perfect example of mutual dependence and symbiotic balance in nature. But detailed observation of the process by C. D. James and co-workers (*Oikos* **69**, 207–216; 1994) shows that the host plant operates some subtle strategies to prevent this apparently servile moth from wreaking havoc with the yucca's seed production.

Pollination of the yucca takes place at night, when the flowers are most strongly scented and the moths are active. The female moth gathers pollen from the anthers of one flower, then visits another where it inspects the ovary to check whether some earlier visitor has already been active at the site. If not, it lays one egg in each locule of the ovary and then deposits a load of pollen into the stigma tube to ensure pollination and consequent ovary development. The larvae subsequently hatch and feed upon the young fruits. Unpollinated flowers wither and

fall, and the developing larvae in the growing fruits mature at different rates, emerging over the course of several years and thus ensuring population continuity through those years when the plant may not flower.

There is one fly in the ointment, however: many of the pollinated flowers and developing fruits with yucca moth eggs or larvae within them also abort and perish. James and his research team have attempted to quantify and assess the importance of this loss to the yucca moth population and have come up with the surprising observation that in almost 12,000 flowers whose history was traced, only 700 produced mature fruit (approximately 6 per cent). This is clearly bad news for the yucca moths and they themselves cannot be blamed for this poor fruit productivity because even hand pollination of flowers by the researchers gave no improvement in fruit yield. A check revealed that 90 per cent of those flowers that had been effectively pollinated still failed to develop. So it cannot be pollination that is limiting fruit production, but more likely resource allocation by the plant. After all that effort on the part of the moths, the yucca jettisons the major part of its developing fruits.

A yucca inflorescence maintains a sequ-

ence of flowering for a period of about two weeks, and James's team observed that only the flowers produced in a five-day window actually manage to produce mature fruits; all the rest abort. Even more remarkable is the unpredictability of when this window will occur during the life of the inflorescence, for it does not seem to coincide with any climatic, temporal or other factor. It is almost as though the plant is throwing random valid cards into a pack of duds, and in this way is ensuring that all the egg-laying female yucca moths are unable to home in on the genuine fruits that could support their young. The selective advantage in such mass random abortions must relate to the control of moth populations and their depredations on the yucca's reproductive resources.

Because figs have a similarly close mutualism with their pollinators, the fig wasps, the question must be asked, do they resort to similar suicidal strategies to control their arthropod pollen-vectors? Apparently not, but there are other factors operating that may ensure that some seeds survive. The fig is a false fruit, a fleshy goblet containing hundreds of flowers, and the sheer abundance of the flowers may in itself ensure that not all of them will be effectively parasitized by the pollinating wasps. Added to this, the female flowers are of two types, long-styled and short-styled, and because the style surfaces form an even carpet over which the wasp wanders, its probing ovipositor is likely to reach only those ovaries topped by shorter styles. The long-styled flowers escape unscathed. Even entrance to a fig may present problems to the fertilized female wasp, as this can be effected only through a terminal pore, or ostiole, which is barricaded by stiff scales that present such a mechanical barrier that many females fail to enter and those that do usually lose their wings in the process. This is the less co-operative face of mutualism. With these restraints in place, the fig does not seem to require more extreme methods for limiting wasp populations: indeed, many fig trees produce non-functional crops of figs that serve simply to ensure the survival of the wasp during the off-season.

The yucca lacks these numerical and mechanical defences. Its ovaries are formed of just three loculi and the flowers are of open access. The subtlety of its survival strategy lies in the unpredictability of its fruit abortion. It is satisfying to the Darwinian mind, however, to discover that the apparently co-operative interaction of these two species, yucca and yucca moth, is based not on mutual altruism but on an aggressive balance of self-interests. □

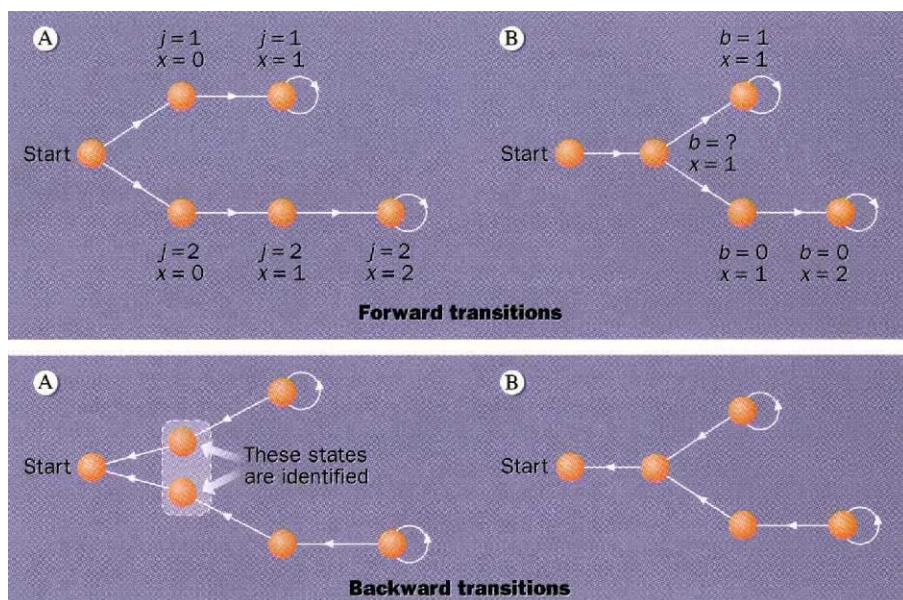
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A question of identity

Annabelle McIver

How much software does it take to rush someone to hospital? Thousands of lines: and system failure, as the London Ambulance Service found out last year, risks lives. Checking huge computer programs by hand is impossible — only a mathematical treatment will guarantee safe working. In the incipient stage of large-scale software development, the design may include abstract data objects such as sets which must be replaced by concrete

programs are deemed to be correct if their behaviours lie in this range — even if they can make arbitrary choices between behaviours at run-time, independently of the inputs. The trick for obtaining a smooth passage from the design goals (or specification) to computer code is to consider both as pieces of formal text which can be interpreted as processes that effect state changes during a computation. In effect both are regarded as programs, the speci-



Are A and B equivalent? Using forward transitions (top) we cannot superimpose B's diagram into A's because A starts by branching whereas B does not. With time running backwards (bottom) A's first choice is irrelevant. The two states with $j=1, x=0$, and $j=2, x=0$ can be 'identified' — collapsed into one — to produce a diagram identical to B's.

data structures more suited to run on computer. The replacement process can be difficult to test rigorously: theories of programming have been unable to prove that some intuitively viable substitutions are correct, particularly when random behaviour is allowed. Now, though, rules have emerged that are universally applicable to all viable substitutions¹⁻³.

For computer programmers, correctness is a grave concern. Their errors are more likely to be detected than those of their purer mathematical cousins, whose work resides comfortably in the domain of the abstract away from the harsh scrutiny of everyday experience. Behind the assertion that a program achieves its design goals lies a proof which will be partially checked every time the program runs. If the proof is invalid for particular conditions then so will the program be, and a random computer user has an uncanny way of finding exactly those spurious conditions.

The specification is a precise description of the acceptable behaviours, and

fication being at the human level and the computer code a translation of it at the machine level. The specification is generally a few pages of standard mathematics, an appropriate environment in which to develop algorithms that work, and in any case much easier to think about than the thousands of pages of code that must replace it. The translation process is called algorithmic refinement, and may require many small steps to accomplish fully.

A crucial part of the development is data refinement. This applies when a program uses self-contained modules and is a rule governing when it is safe to replace one module by another. Each module must guarantee to meet its specification (conditions on its observed variables) but is free to use any manner of hidden variables to achieve it. Thus data refinement is reminiscent of a Turing test: module B can replace module A if any program using the observed variables cannot distinguish between them, and modules with identical external behaviour should be able to be substituted for each

other. Usually B will be code, but the rule applies to all text substitutions.

As an example, suppose that a module must guarantee to increment from zero the observed variable x at least once and at most twice. Any program using the module may examine but not modify x 's value. One way is to use module A, which has hidden variable j . When A runs it initially chooses j 's value at random (either 1 or 2), and then increments x until it equals j . Alternatively the module B with hidden variable b could be used. B first increments x once. It then decides whether to increment again by choosing b 's value (either 0 or 1) arbitrarily. It increments again if b is 0 and not otherwise.

The challenge is to show that B is a valid substitute for A, but with arbitrary run-time behaviour this is not so easy. It boils down to comparing their behaviour. In technical terms it means that any program using the module B algorithmically refines any program using A. To make the comparison precise, imagine that a program's behaviour can be broken down into individual steps, each one observed as a transformation of states (values of program variables). The permitted transformations can be represented in a diagram as arrows connecting pairs of states (see figure). Where choices can be made this will be observed as a branching. Algorithmic refinement just means that B's diagram can be superimposed into A's — in other words any transition step made by B can be made by A.

In the case of data refinement this definition cannot be used directly, because the state spaces are not the same — both B and A have their own hidden variables which must be accounted for. However, we can recover the idea behind algorithmic refinement by considering only the observed behaviour — remember that a program using the module never sees the hidden variables. What we need is the state diagram of the observed variables, and this is obtained from the original diagram by relabelling the states only by the observed variables whilst preserving its shape. B is said to simulate A if we can superimpose B's relabelled diagram into A's, and simulation implies algorithmic refinement.

Unfortunately this analysis is insufficient for all the cases when the diagrams have branching. Recall the example. On initialization A decides, unbeknown to the user, what the final value of x is to be — it is the value assigned to j . The more fickle B delays for as long as possible the decision determining x 's final value, and this uncertainty is seen as a branching when x is already 1, making simulation impossible (see figure).

So the analysis must be sneakier. We turn the clock backwards — instead of looking for transitions starting from a particular state, we look for backward

transitions that end up at a particular state. As for simulation, diagrams of the backward transitions for the modules can be constructed and compared, giving a new relation called co-simulation. This view of programming solves the cases when random choices are made, because the possible choices that were never made become irrelevant. What's more, it turns out that the co-simulation implies algorithmic refinement. In the example, looking back from any final value of x means that only the choice to increment is observed, and this is the same in both A and B. In terms of diagrams, it means that we can safely identify A's first two states — that is, regard them as identical.

In the theory based on diagrams for programs, simulation and co-simulation are different, and both are needed to prove mathematically that all valid substitutions are correct¹. But now, thanks to Paul Gardiner and Carroll Morgan^{2,3}, the story has a far more satisfactory ending.

There is a more general theory of programming (related to the idea of backward transitions) in which programs are associated with predicate transformers, which are more detailed objects than diagrams. The state diagrams are part of this more general setting rather in the way that integers (1, 2...) are embedded into the larger set of rational numbers (1/2, 3/5, 2/1...). The analogy runs deeper. Just as in the rational numbers (but not in the integers) it is possible to divide any number by another, in the more general theory there is an operation analogous to division. Gardiner and Morgan noticed that this 'division' operation makes it possible to combine the notions of simulation and co-simulation — simulation being the 'numerator' and co-simulation the 'denominator'. This compound operator automatically applies both checks for simulation and co-simulation in one go, giving a neat, single characterization of data refinement.

Giving equal status to specification and code brings programming into the mathematical domain in which it truly belongs and out of the wilderness of the 'guess and see' methods. Improvements to refinement rules allow greater freedom of expression whilst maintaining the possibly life-preserving correctness. Placed in this context, the better understanding of program behaviour is likely to turn the process of developing code into a safer ride with no nasty surprises at the end of it. □

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1. Hoare, C. A. R., He Jifeng & Sanders, J. W. *Inform. Proc. Lett.* **25**, 71–76 (May 1987).
2. Gardiner, P. H. B. & Morgan, C. C. *Formal Aspects Comput.* **5**, 367–382 (1993).
3. Gardiner, P. H. B. & Morgan, C. C. *A Single Complete Rule for Data Refinement in On The Refinement Calculus* (eds Morgan, C. & Vickers, T.) (Springer, Berlin, 1994).

DAEDALUS

Walls have voices

A SUPERMARKET bar-code reader has a special speaker that bleeps when the code is verified. Daedalus muses that the reading process should generate a bleep itself. As the laser beam sweeps across the white label, it encounters a sequence of black bars which absorb its energy and convert it to heat. The scan therefore releases a series of heat pulses which, warming up the local air and increasing its pressure, should launch a sound with the waveform of the bar code. The resulting feeble chirp is presumably lost in the hubbub of the supermarket.

DREADCO's physicists are now trying to increase it. One team is adapting a past triumph, a 'blacker-than-black' printing ink that sets to a microfibrous surface like black velvet. This absorbs laser light totally, and transfers its energy very efficiently to the air trapped between the fibres. A rival team is devising an ink foamed with hydrogen, which conducts heat faster and therefore expands more rapidly than any other gas. One of these schemes, or both in tandem, should make possible a bar code which, when scanned by a laser, emits a loud characteristic chirp of its own.

The new sonic ink should find uses far beyond the banal world of retailing. Daedalus sees it as a source of high-fidelity sound. A laser scanning a sonic-ink soundtrack should generate a perfect sound, with no amplifiers or speakers to contribute noise or distortion. At first he envisaged a sort of ultimately simple gramophone, whose soundtrack-coded disk spun under the reading laser and radiated perfect sound into the air. But he then realized that even the spinning disk could be dispensed with. A sonic-ink soundtrack on any wall or ceiling would speak up when scanned from a distance by a suitable laser.

DREADCO's sonic decor will transform audio technology. Public places such as railway stations, airports and shopping malls will bear on their walls snappy advertisements, regularly scanned, as well as useful information and various emergency announcements to be activated only on demand. Sonic road-markings will be scanned by each passing vehicle by means of a scanning laser pointing down at the road. Home owners will happily hang musical sonic-ink soundtracks on their walls, to be swept at will by a neat steerable laser on a coffee table. But a track stretching right along the wall would generate music which drifted along it with the scanning spot. It might be better to fold the track into a compact zig-zag or spiral pattern, print it on a simple poster, and let the laser trace along it by optical feedback.

David Jones

Dentine in conodonts

SIR — Recent work on conodont palaeobiology^{1,2} has provided a substantial amount of data supporting their classification as vertebrates. Some have accepted this hypothesis³, whereas others have not⁴, because of the apparent absence of dentine in conodont elements, a tissue which has been assumed to be universal in vertebrates. Although histological studies have identified vertebrate hard tissues in conodont elements^{2,5}, none has yet conclusively identified dentine in the apatitic feeding structure. We now show evidence for the presence of dentine in conodonts, conclusively demonstrating that this group can be classified as vertebrate.

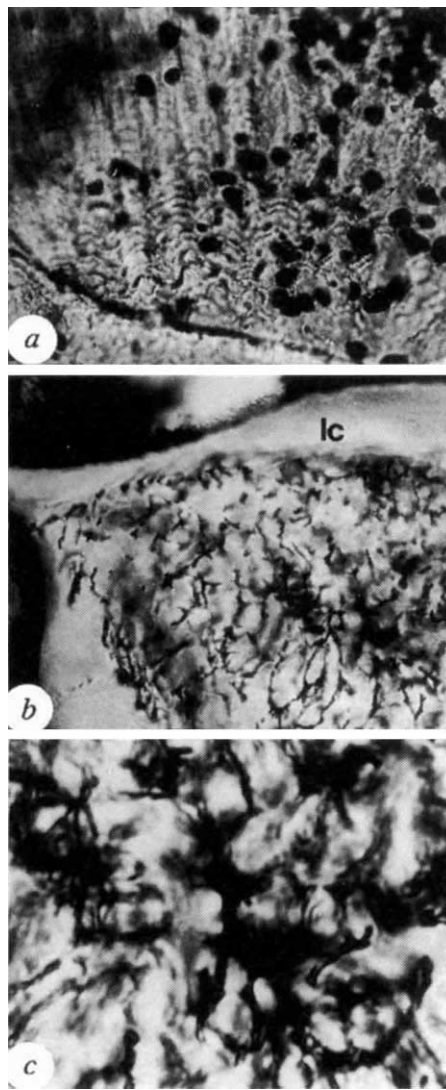
As part of a detailed investigation into the histology of hard tissues in the earliest vertebrates, we examined elements of various conodont taxa from the Late Ordovician (Caradoc) Harding Sandstone Formation of Colorado, a unit also containing at least three accepted early vertebrate genera with which direct histological comparison is possible.

The conodont *Chirognathus* Branson and Mehl has been reconstructed with a siximembrate oral apparatus, and the genus has a typical prioniodinid apparatus⁶. Histologically, the discrete elements are divisible into a crown overlying and enclosing a basal body. Although in many conodonts the element crown is differentiated into lamellar tissue (enamel homologue) surrounding denticle cores of white matter (cellular bone)², *Chirognathus* is representative of hyaline conodonts wherein the crown is formed entirely from the lamellar tissue. However, it is the nature of the underlying basal body upon which we focus here.

We examined double-polished thin sections (approximately 50 µm thick), cut through the basal bodies of *Chirognathus* using polarized and Nomarski differential interference contrast optics together with complimentary electron microscope techniques. The basal tissue in *Chirognathus* is characterized by a series of finely spaced growth lines which are generally scalloped and surround occasional, discrete calcospherites (*a* in the figure). The growth lines are disrupted by tubules (approximately 1 µm in diameter) which run perpendicular to the growth surface and are pervasive throughout the bulk of the tissue, a pattern characteristic of vertebrate dentines⁷. The scale of these features in the basal body of *Chirognathus* falls within the range of size seen in the dentines described from the well documented heterostracomorphs of the Harding Sandstone and its stratigraphic equivalent elsewhere in North America⁷⁻¹¹.

Neocoleodus Branson and Mehl, also from the Harding Sandstone, has been

included within the Family Coleodontidae, although the lack of a clearly recognizable multi-element apparatus makes the ordinal classification problematic⁶. Elements referable to this genus are formed from typical conodont lamellar crown tissue which is underlain by a basal body (*b* in the figure). The basal body is



a, Thin section through an element of *C. duodactylus* Branson and Mehl, from the Harding Sandstone Formation (specimen BU 2251, Lapworth Museum, University of Birmingham, UK), showing the presence of scalloped growth lines with perpendicular traces of tubules. Field width, 50 µm. *b*, Nomarski interference micrograph of a transverse section through an element of *Neocoleodus* Branson and Mehl, from the Harding Sandstone (BU 2257), branching tubules, filled with iron oxide, in the basal body of the specimen appear to have a general alignment perpendicular to the lamellar crown (*c*). Field width, 175 µm. *c*, Nomarski interference micrograph of the basal body core in the specimen illustrated in *b*. The larger rounded structures may represent cell lacunae, and are approximately 5 µm across. Field width, 75 µm.

unlike that identified in *Chirognathus* or those conodonts which we have previously examined histologically². It lacks scalloped growth lines and a calcospheritic pattern, and is instead typified by branching tubules (approximately 1 µm in calibre) infilled by iron oxide during diagenesis (*b*, *c* in the figure). They have a varying alignment approximately perpendicular to the junction with the surrounding crown tissue. This tissue closely resembles the mesodentine found directly under the cap of odontodes referable to 'vertebrate indeterminate A' of Denison¹², both in dimension and appearance of the tubule network. It thus represents an additional tissue found in the basal body of conodont elements.

The identification of diverse dentine types in conodont elements from the Harding Sandstone offers additional and conclusive evidence for the vertebrate nature of conodonts. The presence of two forms of dentine forming basal bodies in the taxa described here contrasts with the putative globular calcified cartilage which has been identified in the same position in elements of *Cordylodus* Pander². It seems that both conodonts and agnathans^{7,9} experimented with many tissue types and tissue associations in the early history of vertebrate biomineralization.

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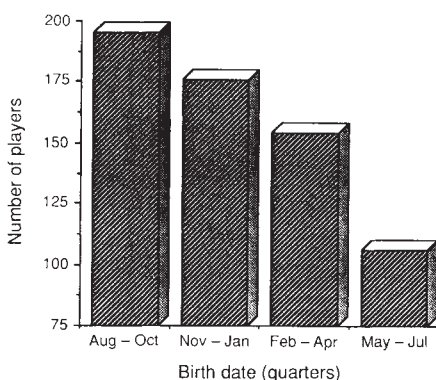
1. Aldridge, R. J., Briggs, D. E. G., Smith, M. P., Clarkson, E. N. K. & Clark, N. D. L. *Phil. Trans. R. Soc. Lond.* **338**, 405–421 (1993).
2. Sansom, I. J. *et al. Science* **256**, 1308–1311 (1992).
3. Briggs, D. E. G. *Science* **256**, 1285–1286 (1992).
4. Forey, P. & Janvier, P. *Nature* **361**, 129–134 (1993).
5. Dzik, J. in *Problematic Fossil Taxa* (eds Hoffman, A. & Nitecki, M. H.) 240–254 (Oxford University Press, New York, 1986).
6. Sweet, W. C. *The Conodonts* (Oxford University Press, New York, 1988).
7. Smith, M. M. & Hall, B. K. *Biol. Rev.* **65**, 277–373 (1990).
8. Ørving, T. *Zool. Script.* **18**, 427–446 (1989).
9. Halstead, L. B. in *Evolutionary Aspects of Neural Crest-derived Skeletogenic Cells in the Earliest Vertebrates* (ed Maderson, P. F. A.) 339–358 (Wiley, New York, 1987).
10. Denison, R. H. *Fieldiana, Geol.* **16**, 131–192 (1967).
11. Halstead, L. B. *Calc. Tiss. Res.* **3**, 107–124 (1969).
12. Smith, M. M. *Science* **251**, 301–303 (1991).

Scientific Correspondence

Scientific Correspondence is a relatively informal section of *Nature* in which matters of general scientific interest, not necessarily those arising from papers appearing in *Nature*, are published. Because there is space to print only a small proportion of the letters received, priority is usually given according to general interest and topicality, to contributions of fewer than 500 words, and to contributions using simple language.

Birth date and sporting success

SIR — I have found a significant relationship between birth date and success in tennis and soccer. In the Netherlands and England, players born early in the competition year are more likely to participate in national soccer leagues. The high incidence of elite athletes born in the first quarter of the competition year can be



Relationship between birthdate and participation rates in Dutch soccer league. Note the ordinate begins at 75 players.

explained by the effects of age-group position.

In organized sport, talent is considered predominantly in terms of physical skills, and the influence of social and psychological factors is often ignored or underestimated¹. Various studies have investigated the psychological characteristics of elite athletes², but none has looked for an

tribution is not uniform ($P < 0.001$) and a regression analysis demonstrates a clear linear relationship between month of birth and number of participants. The dates of birth of 621 players, compiled into quarters, are shown in the figure. This relationship cannot be attributed to the distribution of births in the Netherlands, as this is highly uniform.

We also inspected the distribution of the dates of birth of English football players in league clubs in the period 1991–92 (ref. 3). Birth dates for all players were tabulated by month and compiled into quarters. The results (table) show the significant effect of date of birth on participation rate of soccer players within each of the national leagues, indicating that, as in the Netherlands, significantly more football players are born in the first quarter of the competition year (which starts in September in England).

There is a known relationship between date of birth and educational achievement^{4,5}, implying that the younger children in any school year group are at a disadvantage compared to the older children. Children who participate in sports are also placed in age groups, and my results imply many athletes in organized sports may never get a fair chance because of this method of classification. Very little attention has been drawn to this problem. One of the few studies done in this area analysed the dates of birth of young Canadian hockey players in the 1983–84 season⁶. Players possessing a relative age advantage (born in the months January–June) were more likely to participate in minor hockey and more likely to play for top teams than players in July–December.

More than 20 years ago, this journal

tionship was found⁵ in the Netherlands. Despite this, no action was undertaken to change the educational system. One can only hope that this will not be the case for sports.

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Sensory maps in the human brain

SIR — Until a decade ago, it was widely believed that once neural pathways were laid down in fetal life or early infancy, little subsequent alteration of these pathways occurred in the adult human brain. Over the past decade, however, shifts of cortical sensory function in adult primates of 1–3 mm following deafferentation have been documented¹. Pons *et al.* recently reported² that facial touch stimuli ipsilateral to a chronic upper limb deafferentation in adult macaques evoked responses of cells in the somatosensory S1 hand representation area with expansion of the facial cortical map by 10–14 mm. V. S. R. *et al.*^{3–5} studied perception in human arm amputees and found that tactile stimuli applied to the lower facial region ipsilateral to the amputation evoked topographically organized, modality-specific, referred sensations in the phantom limb. While such psychophysical findings in amputees and in neurological patients⁶ suggest a functional reorganization of the human somatosensory areas analogous to the physiological changes observed in macaques², more direct evidence is needed.

For this purpose we developed a method of high-resolution, non-invasive somatosensory mapping, based on a magnetoencephalographic (MEG) approach already used and validated for pre-surgical mapping of brain somatosensory regions⁷. Our approach provides highly reproducible, detailed and accurate localizations of discrete areas of the face, hand and arm⁸.

A 37-channel Magnes biomagnetometer (Biomagnetic Technologies, Inc., San Diego) was used to record somatosensory responses evoked by painless tactile stimuli from two adult, human amputees (D.S. and F.A.) and four normal controls. Source localizations were calculated using a single equivalent current dipole (ECD) model^{7,8}. D.S. was studied 8 years after traumatic avulsion of his left brachial plexus, and F.A. 11 years after accidental amputation of his right forearm 8 cm below the elbow. A detailed sensory homunculus was observed bilaterally in all four controls and on the unaffected hemisphere in both amputees. On the affected hemisphere, D.S. showed

PARTICIPATION RATES IN ENGLISH SOCCER LEAGUES

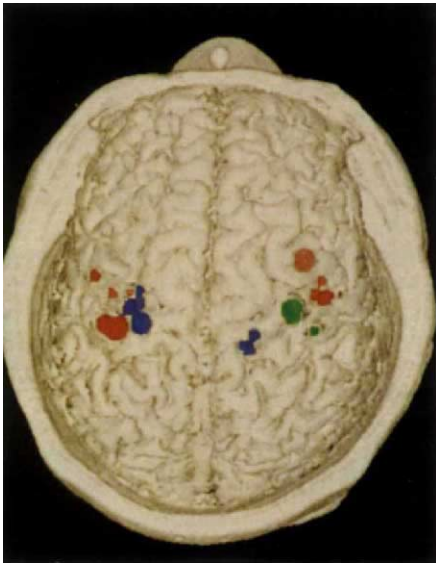
League	Players in birthdate quarters				Statistics		
	Sep–Nov	Dec–Feb	Mar–Apr	Jun–Aug	Total	χ^2	Sig.
FA premier	288	190	147	136	761	75.53	$P < 0.0001$
Division 1	264	169	154	147	734	48.47	$P < 0.0001$
Division 2	251	168	123	131	673	61.11	$P < 0.0001$
Division 3	217	169	121	102	609	52.38	$P < 0.0001$
Total	1,020	696	545	516	2,777	230.77	$P < 0.0001$

effect of age. I discovered a strikingly skewed distribution of the dates of birth of 12- to 16-year-old tennis players in the top rankings of the Dutch youth league. Half of a sample of 60 tennis players were born in the first 3 months of the year.

This discovery led me to consider the distribution of the dates of birth of professional soccer players. In the Netherlands, there are two leagues comprising a total of 36 clubs. I found a striking difference between participation rates of those born in August and July. The Dutch soccer competition year starts on the first of August. A χ^2 test indicates that the dis-

tribution is not uniform ($P < 0.001$) and a regression analysis demonstrates a clear linear relationship between season of birth and cognitive development⁷. The authors attributed this relationship to a fault in the British educational system. A similar rela-

1. Dudink, A. *Eur. J. High Ability* **1**, 144–150 (1990).
2. Dudink, A. & Bakker, F. *Ned. Tsch. Psychol.* **48**, 55–69 (1993).
3. Rollin, J. *Rothmans Football Yearbook 1992–93* (Headline, London, 1992).
4. Shearer, E. *Educ. Res.* **10**, 51–56 (1967).
5. Doornbos, K. *Date of Birth and Scholastic Performance* (Wolters-Noordhoff, Groningen, 1971).
6. Barnsley, R. H. & Thompson, A. H. *Can. J. Behav. Sci.* **20**, 167–176 (1988).
7. Williams, Ph., Davies, P., Evans, R. & Ferguson, N. *Nature* **228**, 1033–1036 (1970).



Top view of a combined MEG and three-dimensional surface-rendered MRI of patient F.A. The unaffected right hemisphere shows the primary somatosensory face area (red) lateral, anterior and inferior to the hand localizations (green), which are in turn lateral, anterior and inferior to the upper arm region (blue). The affected left hemisphere shows the face (red) and upper arm region (blue) extending into the expected hand territory.

a significant superior intrusion of facial localizations into the hand territory as compared with the controls ($P < 0.02$). The affected hemisphere of F.A. exhibited superomedial intrusion of facial and inferolateral intrusion of upper arm localizations into the expected hand region (see figure). Compared with controls, a significant contraction of the distance between upper arm and facial localizations was seen on F.A.'s affected hemisphere ($P < 0.01$). Comparison of mirror-image facial localizations on the affected versus unaffected hemispheres of D.S. and F.A. showed displacements of up to 35 and 30 mm, respectively.

Psychophysical testing revealed two maps of referred sensations in both amputees, one on the face and another on the upper arm, reflecting the fact that the hand area in the Penfield homunculus is flanked by the face and arm. Expansion of face and upper-arm representations into the cortical hand area would produce both the observed clustering of facial and upper-arm MEG localizations, and the clustering of points on the skin of the face

and upper arm which yield referred sensations in the phantom hand.

These results provide the first direct demonstration of massive reorganization of sensory maps in the adult human brain⁹, an observation which correlates well with physiological work on macaques² and psychophysical experiments on humans³⁻⁵. We conclude that new patterns of precisely organized and functionally effective connections can emerge in the adult human brain. Understanding these phenomena would have therapeutic implications, both for recovery from brain injury and for treatment of phantom limb pain.

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New mammals not so seldom

SIR — An item in *Nature's* contents panel¹ suggests we should expect only "obscure microbes and insects" to turn up as new species and refers to the bovid *Pseudoryx nghetinhensis* as "the first large mammal to emerge since the kouprey" (named in 1937). In his News and Views comment, Henry Gee² claims the Chacoan peccary, not the kouprey, was "the latest and last large mammal to have been described." Dung *et al.*³, in their paper naming *P. nghetinhensis* in the same issue, say that it "has been more than 50 years since any comparable find of a large mammal species . . . the last being the kouprey." But during the period 1937 to the present, at least 16 large mammals have been discovered as living species, three of which also represented undescribed genera. Thus there have been about three new species of large mammals discovered per decade.

The species are⁴: two porpoises — *Lagenodelphis* (new genus) *hosei* (1956) and *Phocoena sinus* (1958); four beaked whales — *Tasmacetus* (new genus) *shepherdi* (1937), *Mesoplodon ginkgodens* (1958), *M. carlhubbsi* (1963), *M. peruvianus* (1991); a wild pig — *Sus heurni* (1987); the Chacoan peccary — *Catagonus wagneri* (named from bones from Indian mounds in 1930, present-day animals collected as early as 1936 (ref. 5), first reported as a living animal in 1975); four deer — *Mazama chunyi* (1959), *Moschus fuscus* (1981), *Muntiacus atherodes* (1982), *Muntiacus gongshanensis* (1990); the kouprey — *Bos sauveli* (1937); a gazelle — *Gazella bilkis* (1985); a wild sheep — *Pseudois schaeferi* (1963); and a

'bovid' — *Pseudoryx* (new genus) *ngheetinhensis* (1993).

The first-mentioned porpoise, all four of the beaked whales, and the kouprey are much larger and heavier than *Pseudoryx nghetinhensis*⁶. The last 10 listed above are in the order Artiodactyla, and the last four are bovids. Not everyone necessarily agrees or will agree in future that all these animals deserve full specific status, but certain other large mammals (if any) named during this period and currently not treated as full species may come to be so regarded. The number named since 1937–1993 and that will be recognized at various times in the future will probably not deviate far from 16.

Varying time intervals can elapse between one or more specimens of a new species first becoming available, some knowledgeable scientist(s) seeing them, realization that the undescribed species exists, and eventual publication. As to the dates of these events, the year of publication of a new name is usually the best documented and therefore is treated here as the year of "discovery". In the case of *Gazella bilkis*, at least 116 years elapsed between the first specimen becoming available and publication of the new name⁷. In the case of *Tasmacetus shepherdi*, this interval appears to have been four years⁸; for *Bos sauveli*, seven⁹; for *Phocoena sinus*, eight¹⁰; for *Mesoplodon peruvianus*, fifteen¹¹.

Because most mammals are small, most now being named as new species are small. Since 1930, or 27% of the time (1758 to the present) that mammals have been scientifically named, there have been 742 recognized species of living mammals named⁴. This is 16% of the 4,629 species known (including some based only on subfossils)⁴. The numbers named per year for each decade are as follows: about 16 per year (1930s); 7 (1940s); 10 (1950s); 9 (1960s); 11 (1970s); and 16 (1980s). More than 46 have already been named in the 1990s. Thus there is no end in sight. These figures are remarkable in view of the supposed contemporary de-emphasis on "alpha" taxonomy in zoology and the undoubted accelerating extinction of species before discovery.

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1. *Nature* **363**, no. 6428, v (1993).
2. Gee, H. *Nature* **363**, 398 (1993).
3. Dung, V. V. *et al.* *Nature* **363**, 443–445 (1993).
4. Wilson, D. E. & Reeder, D. M. (eds) *Mammal Species of the World: A Taxonomic and Geographic Reference* (Smithsonian Institution, Washington, DC, 1993).
5. Wetzel, R. M. & Crespo, J. A. *Rev. Mus. Argent. Cienc. "Bernardino Rivadavia"*, **Zool.** **12**, 25–26 (1975).
6. Nowak, R. M. *Walker's Mammals of the World* (Johns Hopkins Univ. Press, Baltimore, 1991).
7. Groves, C. P. & Lay, D. M. *Mammalia* **49**, 27–36 (1985).
8. Oliver, W. R. B. *Proc. zool. Soc., London* **107**, ser. B 371–381 (1937).
9. Hoffmann, R. S. *Mammalia* **50**, 391–395 (1986).
10. Norris, K. S. & McFarland, W. N. *J. Mammal.* **39**, 22–39.
11. Reyes, J. C. *et al.* *Mar. mammal Sci.* **7**, 1–24.

1. Merzenich, M. M. *et al.* *Neuroscience* **10**, 639 (1983).
2. Pons, T. P. *et al.* *Science* **252**, 1857–1860 (1991).
3. Ramachandran, V. S. *Proc. natn. Acad. Sci. U. S. A.* **90**, 10413–10420 (1993).
4. Ramachandran, V. S., Stewart, M. & Rogers-Ramachandran, D. C. *NeuroReport* **3**, 583–586 (1992).
5. Ramachandran, V. S. *et al.* *Science* **258**, 1159–1160 (1992).
6. Halligan, P. W. & Marshall, J. C. *Nature* **361**, 410 (1993).
7. Gallen, C. C. *et al.* *Neurosurgery* **33**, 260–268 (1993).
8. Yang, T. T., Gallen, C. C., Schwartz, B. J. & Bloom, F. E. *Proc. natn. Acad. Sci. U. S. A.* **90**, 3098–3102 (1993).
9. Yang, T. T., Gallen, C. C., Ramachandran, V. S., Cobb, S. & Bloom, F. E. *Soc. Neurosci. Abstr.* **19**, 162 (1993).

No verification for Milankovitch

SIR— We were puzzled by the table in the Scientific Correspondence¹ by Emiliani. He rejects the conventionally used terminations (glacial-interglacial transitions) as time markers and focuses on bathythermals (the coldest portions of glacial cycles), which he deems to be sharper and therefore more precise time markers. He claims that bathythermals in the Devils Hole $\delta^{18}\text{O}$ chronology² occur at times when the orbital parameters of obliquity and eccentricity are both “low”, as determined from Berger’s³ figures, thereby supporting the Milankovitch mechanism¹.

Unfortunately, Emiliani does not specifically define what he means by the critical terms “low” or “when they approach coincidence”, but we assume he takes “low” to mean the times when both obliquity and eccentricity were at a minimum, or obliquity was at a minimum and eccentricity was less than at least the long-term (0–600,000-year) average value. We show in the figure the seven astronomical “low” events that Emiliani gives in the third column in his table, as well as the seven (but not identical) events that satisfy the specific definition of astronomical low conditions using data in ref. 4. We were puzzled as to why Emiliani omitted the two well defined “low” events at 395,000 and 517,000 years and note that they do not correspond to bathythermals in either the Devils Hole² or the marine⁵ $\delta^{18}\text{O}$ chronologies. Indeed, the 395,000-year “low” event occurs during a peak interglacial time. We also note that Emiliani’s designation of a “low” event at 555,000

and 150,000 years does not fit the earlier stated definition.

Also shown in the figure are the eight major $\delta^{18}\text{O}$ minima, denoting times of full glacial climate, found in the Devils Hole chronology, and the subset of six events that Emiliani gives in the second column in his table. He did not mention the two Devils Hole isotope minima at 223,000 and 173,000 years, which do not correspond to any astronomical “low” event.

In comparing the astronomical “low” events predicted by the specific definition with the minimal isotope events found in the Devils Hole chronology, one sees that although there are four ‘matches’, there are six ‘non-matches’, twice when a bathythermal would be predicted but did not happen, and four times when one did occur but not during an astronomical “low” event. Thus the astronomical condition that Emiliani specifies is neither sufficient nor necessary for the occurrence of bathythermals.

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1. Emiliani, C. *Nature* **364**, 583 (1993).

2. Winograd, I. J. *et al. Science* **258**, 255 (1992).

3. Berger, A. *Quat. Res.* **9**, 139 (1978).

4. Berger, A. & Loutre, M. F. *Quat. Sci. Rev.* **10**, 297 (1991).

5. Imbrie, J. *et al. in Milankovitch and Climate* (eds Berger, A. *et al.*) 269–305 (Reidel, Dordrecht, 1984).

Skin cancer and ultraviolet

SIR— Madronich and de Gruijl¹ provide up-to-date estimates of total column ozone depletion by latitude for the 14-year period 1979 to 1992, and estimates of corresponding increases in ground-level ultraviolet radiation with reference to the action spectra for erythema induction, DNA damage and non-melanocytic skin cancer. These are informative and useful estimates.

They also estimate increases in incidence of basal and squamous cell carcinomas of the skin that may result from the increases in ultraviolet radiation. But there is an inevitable delay following ultraviolet exposure before consequent non-melanocytic skin cancer develops in humans. Although the duration of this delay is not known, it is probably not less than about 20 years at its minimum. This minimum can be inferred from the fact that the incidence of these cancers is very low before 20 years of age² although exposure to solar ultraviolet begins, for most people, soon after birth. Further, it will probably be necessary for the generation experiencing the increase in ambient ultraviolet in childhood to have reached old age before new ‘steady state’ incidence rates of skin cancer, such as those esti-

mated by Madronich and de Gruijl, are reached. This is suggested by evidence that about 80% of skin cancers in Australia can be attributed to exposure to the sun in the first 10 years of life³.

Ozone-layer depletion will almost certainly increase skin cancer incidence by proportions of the order estimated by Madronich and de Gruijl. These increases, however, may not yet have begun and would not be expected to reach their maxima, even if there is little further increase in ambient ultraviolet for another 70 or 80 years.

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MADRONICH AND DE GRUIJL REPLY— Extrapolating trends and effects of ultraviolet into the future is very hypothetical due to uncertainties that arise from atmospheric chemistry, epidemiology, and related disciplines. The values that we calculated¹ are one plausible measure of the magnitude of the ozone-ultraviolet effects. They represent the steady state increase in non-melanoma skin-cancer rates resulting from a permanent decrease in ozone as large as that observed in 1979–92. The timescales for atmospheric change and skin-cancer development are still far from certain: ozone reductions are expected to continue well into next century⁴, and the time between ultraviolet exposure and development of skin cancer is essentially unknown, as already recognized by Kriker *et al.* Clearly, there will not be an instantaneous transition to a new steady state, but the assumptions necessary to predict the temporal evolution cannot be justified by present knowledge and would be more hypothetical.

The indication that early-life exposure does more damage is thought-provoking: although lessening the concerns for many, it also suggests that younger and future generations may be at a quantitatively higher risk in relation to atmospheric ozone trends. One should be aware, however, that most people (especially indoor workers) receive much of their lifetime ultraviolet exposure during childhood.

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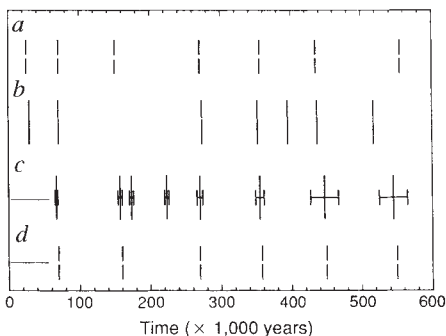
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1. Madronich, S. & de Gruijl, F. R. *Nature* **366**, 23 (1993).

2. Parkin, D. M. *et al. Cancer Incidence in Five Continents* Vol. VI. (Int. Agency Res. Cancer, Lyon, 1992).

3. Kriker, A. *et al. Int. J. Cancer* **48**, 650–662 (1991).

4. *Scientific Assessment of Ozone Depletion WMO Ozone Rep.* **25** (1991).



Comparison of all times satisfying the definition of an astronomical low event (see text) and times when Devils Hole $\delta^{18}\text{O}$ minima occurred, with the respective values from columns 3 and 2 of the table in ref. 1. Note that the Devils Hole chronology does not cover the period 60,000 to 0 years indicated by the horizontal lines. a, Emiliani’s astronomical low events; b, times when obliquity is minimal and eccentricity is either minimal or below average; c, Devils Hole $\delta^{18}\text{O}$ minima ($\pm 2\sigma$); d, Emiliani’s Devils Hole ice ages.

Good food, fine words

Nicholas Kurti

Food and Feast in Medieval England. By P. W. Hammond. *Alan Sutton: 1993. Pp. 169. £16.99.*

History of Food. By Maguelonne Toussaint-Samet. *Blackwell: 1993. Pp. 801. £25.*

A Taste of History: 10,000 Years of Food in Britain. By Peter Brears, Maggie Black, Gill Corbishley, Jane Renfrew and Jennifer Stead. *English Heritage/British Museum Press: 1993. Pp. 349. £14.95.*

Les Secrets de la Casserole. By Hervé This-Benckhard. *Editions Belin: 1993. Pp. 222. FF110.*

It is a pleasant task to review four books on food that may be regarded as four courses of a tasty and satisfying meal. The comparison also helps the reviewer to organize his work.

We begin with an appetizer or starter. P. W. Hammond's book is certainly no *hors d'oeuvres variés*, no varied titbits but a neat, compact scholarly volume on food in England covering the period 1250–1550. The first chapter is entitled "Where the Food came from", and having read it one tends to agree with the opening sentence: "Food in the Middle Ages was not so different from food today". This does not mean that meals were similar to ours; the author wisely announces that, not being a cook, he did not cover cooking or household management. There follow three chapters on the food of the "country-man" (labourers, tenants, villagers and so on), "town dweller" and "gentry", the latter including the church and the aristocracy. The author presents a balanced view of the role of food in the life of the English people and its economic and social aspects, with glimpses of the relationships between the various classes.

A chapter on "Adulteration and Nutrition" contains a lot of fascinating information. We learn that such was the opposition to the introduction of beer from Holland in the fifteenth century that in 1512 the use of hops, a "poisonous weed", was prohibited. Late meals were to be avoided and, "if sleep was essential after eating, it should be done leaning against a cupboard". (Not an ideal siesta!) Reading about "Table Manners", one gets the impression that meals in those days were more civilized than some films have led us to believe. Finally, the chapter on "Feasts" proves that they were the forerunners of modern banquets, which offer a lot to the eyes and the ears but little to the palate.

This well-illustrated book with an up-to-date bibliography of 150 titles is a modest but worthy complement to J. C. Drummond and Anne Wilbraham's *The Englishman's Food* (Cape, 1957/Pimlico, 1992).

Maguelonne Toussaint-Samet's book is an impressive main course: 800 pages of history, anthropology, mythology, philo-

sophy, divinity, economics, social sciences and even some natural sciences. One way to discover the author's intentions is to look at the table of contents, which lists nine main sections, the first two dealing with "Collecting, Gathering, Hunting"

and "Stock-breeding, Arable Farming: Meat, Milk, Cereals". This gives the impression that the author has organized her work along historical lines as the various foodstuffs were discovered and came into use. But the next section brings a new principle into play: we have oil, bread and cakes and wine under the title "The Sacramental Foods". Fish and poultry appear thanks to "The Economy of the Markets" while "Luxury Foods" embraces "treasures of the sea" (caviare, shellfish and so on) and the "treasures of the forest" (pork, *charcuterie*, *foie gras*, truffles). Salt, spices and herbs belong to the "Era of the Merchants", and "New Needs" were satisfied by the "lure of sugar", "coffee and politics", "tea and philosophy" and "chocolate and divinity". (It is surprising in this connection that the author does not discuss why four great English chocolate enterprises belonged to Quakers: Cadbury, Fry, Rowntree and

Terry. Was it their desire to replace alcohol and coffee by something soothing or to prove that humane treatment of slaves in the African cocoa plantations does not vitiate profits?). And so we come to the penultimate section with the simple title "Orchards and Kitchen Gardens", in which the author admirably describes the origins, development and uses of some 30 fruits and vegetables.

This is a remarkable book, full of information culled from serious research. What makes it particularly attractive for browsing is that it is rather undisciplined, as the previous observations have shown. It is rich in anecdotes, in telling *aperçus* and unexpected revelations. Should we pity the geese being fattened up for *foie gras*? They seem to be predisposed to cramming: according to the ancient Egyptians, their ancestors, the wild geese,

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DECEMBER—"A Swallow at Christmas" (*Rara avis in terris*)

crammed themselves in order to have sustenance for their migration to the north. It is also good to be reminded of the chef who prepared *pommes frites* for the luncheon of King Louis-Philippe and his entourage following their journey on the first ever train from Paris to Saint Germain. On being told that the train was late, the chef removed the potatoes from the oil, and when the train arrived he fried them again: they doubled in size and became *pommes soufflées*. Trust the French to create a culinary miracle out of a late train!

For the pudding or sweet course (for 'dessert' see later), I offer *A Taste of History*, a mouth-watering and thought-provoking collection of essays by five of the UK's foremost food-writers/historians. Our appetizer looked at a small chunk of English history; here we have the story of food in Britain since prehistoric times. Hammond's book told us only what

foodstuffs the mediaeval English used for their meals, but in this book, thanks to the many recipes, we also get an idea of what people ate in various periods.

In the first two chapters, Jane Renfrew describes with the help of excellent illustrations how archaeology sheds light on daily life in prehistoric and Roman Britain. "Hokka muggies" made of fish stomach, cod liver and oatmeal is of rather doubtful attractiveness but "slott" (cod-roe dumplings in modern terminology) seems to be worth a try. The recipes of Roman Britain based on Apicius are sophisticated by any standards, such as milk-fed snails "fattened to the point that they cannot return to their shells and then fried" and an omelete made of four eggs, half a pint of milk and one tablespoon of olive oil.

Maggie Black tells us about mediaeval Britain and, as in other chapters, we learn a lot about food resources and choices, cooking methods, techniques and utensils, and table service and etiquette.

Guided by Peter Brears we look around Tudor and seventeenth-century Britain and come almost uncomfortably close to the present day with a tapster (barman) and a cook complaining about the prohibition of Sunday trading. The "rich mince pie" (one-and-a-half pounds of mutton, beef or suet, six ounces of mixed dried fruit) may not be everyone's choice, but if French purists enjoy *canard à l'orange*, why not meat and dried fruit?

In her chapter on Georgian Britain, Jennifer Stead tells us about the introduction of afternoon tea when the main meal moved from 2-3 pm to 6-7 pm, and her section modestly entitled "Ingredients" is full of interesting information about, for example, the use of nutmeg as an all-purpose flavouring.

Maggie Black's second contribution is on Victorian Britain; a clear, concise and scholarly resumé of the many changes that took place during the century of Eliza Acton, Isabella Beeton, Charles Elmé Francatelli and Alexis Soyer, and there are 23 well-chosen recipes for all seasons, from "dinner parties" to "charity foods".

Gill Corbishley's chapter on the twentieth century is for some readers ancient history, for others living memory. It is good to be reminded of what shopping was like earlier this century, of Lyons Corner Houses and teashops and how they gave way after the Second World War to espresso bars, oriental restaurants, take-aways and McDonald's. The recipe section gives, for the pre-1939 period, a prizewinning fish recipe called "Tasty and Cheap"; for the war years we have Lord Woolton's Pie; and the post-1950 period is characterized by *canard à l'orange* and "Imam Bayildi".

This is a delightful book, easy to read, pleasant to look at, much to learn from and a splendid selection of recipes with

which to dazzle, amuse, astound or tease one's guests.

And now we come to our 'English' dessert, which is becoming rare nowadays. After the meal has ended with a pudding or a savoury dish, the table is relaid and various wines such as port or Madeira circulate, accompanied by fruit, nuts and biscuits. This is the time for lively and light-hearted conversation, and what book could better represent this part of a meal than *Les Secrets de la Casserole* written by a young Frenchman. Hervé This-Benckhard is a chemist by training and the associate editor of *Pour la Science*, the French version of *Scientific American*, and, as his book shows, he is also a practising and enthusiastic cook. Noticing that cookery books, old and new, are full of instructions and culinary tricks given without any reason or explanation, he decided to study these cases, mostly experimentally but also by simple reasoning. The result is this book.

The author examined 40 cases, and the following selection of the topics indicates their variety: mayonnaise, soufflés, braising, tenderizing, pressure-cooking, foam, mousses, creams. Often he found the explanation, sometimes he did not. But for him, the search goes on and he ends with a list of 20 questions and asks whether the reader has the answers. So far, the reader who has no French is excluded from this challenge. One can but hope that there will be before long an edition in English that could be called "The Saucepan Mysteries" and have as its motto Kipling's lines from his *Just So Stories*:

I keep six honest serving-men
(They taught me all I knew)
Their names are What and Why and When
And How and Where and Who.

I send them over land and sea,
I send them east and west;
But after they have worked for me,
I give them all a rest.

But not Hervé This-Benckhard. The final sentences of his book are: "Do you know the answers to these questions? You would oblige me by passing them on to me. Do you have other questions? Let me know and I will look for the answers. Meanwhile: BON APPETIT." □

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■ "The discovery of a new dish does more for the happiness of mankind than the discovery of a new star", wrote the French magistrate, politician and gourmet Jean-Anthelme Brillat-Savarin in *The Physiology of Taste* (1825). Hailed by many as the greatest work on food ever published, this charming book is now reissued by Penguin at £7.99. The translation and introduction are by Anne Drayton.

Kids' stuff

David Hawkridge

The Children's Machine: Rethinking School in the Age of the Computer. By Seymour Papert. *Harvester Wheatsheaf*: 1994. Pp. 241. £12.50, \$22.50 (pbk).
Beyond Technology's Promise: An Examination of Children's Educational Computing at Home. By Joseph B. Giacchino, Jo Anne Bauer and Jane E. Levin. *Cambridge University Press*: 1993. Pp. 244, £30.00, \$49.95 (hbk); £11.95, \$16.95 (pbk).

SEYMOUR Papert's first important book, *Mindstorms: Children, Computers and Powerful Ideas* (Harvester, 1982), caught the imagination of tens of thousands of teachers. Unlike almost all others writing about how computers might transform education, he has a strong, consistent vision of children learning by making, including making with computers. He is a source of fundamental ideas.

Papert arrived at the Massachusetts Institute of Technology in 1963 from Geneva, where he had worked with Jean Piaget. Over two decades, he developed, tested and propagated the programming language Logo that enables children to control computers. He also developed a cognitive theory of learning that is a variation of constructivism, a theory defined broadly as learning by constructing one's own knowledge and elaborated in *Constructionism* (Ablex, 1991), which he edited with Idit Harel.

A mathematician by training, Papert portrays himself as a pioneer, along with the psychologist Patrick Suppes and the physicist Jack Kemeny, who originated the programming language BASIC. Suppes, like Papert a skilful entrepreneur, established the Computer Curriculum Corporation, now part of the Paramount conglomerate and selling mass computer-assisted instruction to schools in the United States and even in the United Kingdom. But Suppes' rigid 'instructionism' clashed with Papert's 'constructionism'. Suppes told Papert: "I would rather be precisely wrong than vaguely right".

In *The Children's Machine*, Papert blows a further blast on his war-trumpet. His writing is eloquent, rich in anecdotes, rhetorical and persuasive. He likes to tell stories about himself, his colleagues, opponents and students — and most of all about the children who use his inventions to learn. But he is far from vague in presenting his intellectual arguments. He begins by lucidly recapitulating the old ones, covering new ground only in the middle chapters.

If Logo was Papert's first major contribution, Logo with Lego is his second.

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Between electrode and solution

Allen J. Bard

Fundamentals of Electrochemistry. By Vladimir Sergeevich Bagotzky. Plenum: 1993. Pp. 589. \$138.

ALTHOUGH electrochemistry is an old field, it remains vital because of its usefulness in elucidating mechanisms of organic and inorganic reactions, its many applications and the availability of new techniques for providing fresh insight into the nature of the interface between electrodes and solutions. Interest in the field has also been sparked by the notorious cold fusion episode, which revealed a lack of basic knowledge by most scientists of even the fundamentals of electrochemical systems. The understanding of electrochemistry requires a good background in many areas, including electrostatics, solid-state physics, mass transfer and thermodynamics; an author of a general monograph in this area must guess which topics will already be familiar to readers. Bagotzky opts to treat many of the background topics, but then excludes many of the more modern aspects of electrochemistry.

This book is a translation of a 1988 Russian edition with the addition of brief chapters on photoelectrochemistry, electrokinetic processes and bioelectrochemistry. The treatment of fundamentals, up to about 1960, is well done and the writing is clear. The traditional topics of a physical electrochemistry text are here: properties of electrolytes and interfaces, electrochemical kinetics and applications. Russian work is, of course, well represented, sometimes to the exclusion of more modern work in the same area: for example, in treating electrode potentials in nonaqueous electrolytes, the 1947 proposal of Pleskov is discussed; but later proposals, such as the use of the ferrocene couple as a standard (an IUPAC recommendation), are not mentioned. Similarly, in the discussion of the theory of water and ionic solutions, recent theories based on molecular-dynamics treatments and activity coefficients (Pitzer) are lacking.

Those interested in using electrochemical methods to characterize chemical systems will probably be disappointed with Bagotzky's approach. Although the use of steady-state polarization (current-potential) curves is developed clearly and rigorously, the discussion of the widely used and more powerful transient techniques, such as cyclic voltammetry, is inadequate — the single cyclic voltammogram that appears in the book looks awful. The author also fails to treat many of the modern methods that have been applied to obtain a better understanding of elec-

Techno-toys — thinking about computers in school.

He is now the Lego Professor for Learning Research. He explains how he has taught the basic principles of cybernetics, especially 'feedback', to children so that they can build robot-like Lego models. They program the models, using Logo, to seek light, avoid objects, respond to sounds and so on. Here is the latest kind of active learning: children learning how to control robots' behaviour and to think cybernetically. If this sounds excessively mechanistic, it is not. Papert couches his theory in a deeply caring and humanistic view of children.

The Children's Machine will sell to parents as well as teachers. It will be widely read, as *Mindstorms* was, although maybe not right to the end, where the passion fades. The author's subdued plea in the last chapter for small alternative schools, set up perhaps by half a dozen teachers declaring independence from School, that dreaded formal education system, makes a feeble ending after the early drama and rhetoric. More than a million children use Logo, claims Papert, yet he sounds like a prophet, crying in the wilderness: if only teachers would repent, learners would be redeemed. He won't ask teachers whether Logo works, because they belong to School and they don't understand computers, still less constructionism. Papert's humanism deserts him, perhaps, when he writes about teachers. Instead of talking to them he would rather they read his book.

By contrast, *Beyond Technology's Promise* has little to offer to parents, teachers or other readers. Joseph Giacquinta is a professor of education at New York University. With his two assistants, he has written an over-engineered account of a qualitative study of how children use computers for education at home. His team surveyed and observed 70 computer-using New York families between 1984 and 1986.

Can we still learn something from these wordy, unsurprising findings, published more than six years later? Probably not much. Giacquinta and his team uncovered

sporadic educational use, in which parents did not assist or encourage their children. They conclude: "The educational promise of the home computer was not being fulfilled".

But who promised what? No doubt salespeople made similar promises on behalf of the telephone, gramophone, tape recorder and camera. Since 1986, personal computers have improved and ownership has risen sharply, with huge numbers of people gaining access to networks. But why should children want to use the machines for education rather than hobbies and games? For most kids, the desktop computer is just like the telephone on the wall or the television in the lounge: to be used as and when needed, but not particularly for education.

If Giacquinta met Papert, he would have to admit that *Beyond Technology's Promise* displays an abysmal lack of understanding of Logo. The most perceptive comment in the book is made by a parent: "I sat back and watched [my child] and thought, 'That's a cognitive activity.' I can't think of a parallel activity without the computer that she does . . . And she does this for fun". □

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New Journals Issue

This year, *Nature's* annual new journals review supplement will appear in the issue of 29 September. Publishers and learned societies are invited to submit journals for review. Journals that first appeared during or after June 1992 and issued at least four separate numbers by the end of April 1994 will be considered. Frequency of publication must be at least three times a year. For further information please see last week's issue of *Nature* (7 April), or contact Peter Tallack, *Nature*, 4 Little Essex Street, London WC2 3LF, UK, 071-836-6633 (011-44-71-836-6633 from the United States), extension 2414.

trode surface processes, and, more recently, even an atomic-level view of the electrode surface; spectroscopic methods are dismissed in a single paragraph and other techniques, such as scanning tunnelling microscopy, are not mentioned.

Overall then, if one is looking for a good treatment of classical physical electrochemistry and its traditional topics, this book is a good alternative to several others of a similar type. Unfortunately, the usefulness of the volume is marred by a total lack of references in the text to any of the original literature (there is, however, a bibliography of general monographs and reviews). Readers with a good background in physics and physical chemistry will probably prefer a more chemical approach with greater attention to modern methods. □

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Chance and necessity

David Goodstein

The Refrigerator and the Universe: Understanding the Laws of Energy. By Martin Goldstein and Inge F. Goldstein. Harvard University Press: 1993. Pp. 433. \$29.95, £23.95.

IN a world rapidly running out of fossil fuel, the second law of thermodynamics may well turn out to be the central scientific truth of the twenty-first century. And yet, even though it was discovered 150

years ago, it is well and truly understood by only the tiniest handful of the globe's five billion inhabitants. Clearly, there is much need to spread the word. We have here an attempt to do that.

The Refrigerator and the Universe certainly has its heart in the right place, to say nothing of its nouns and verbs. Its title brings to mind the fact that the second law, discovered by Nicolas Carnot while contemplating a sooty, clanging steam engine, applies even to black holes. Shouldn't an epic story like that be worth a great book? The answer is yes, and I have long thought a book telling it was badly needed. But this, alas, is not the one.

The Goldsteins really really want to teach us thermodynamics so that we can marvel with them at all its beautiful subtleties and consequences. The trouble is that before we can really really understand thermodynamics, we first have to understand a few other things, such as mechanics, energy, kinetic theory, statistics And so we have to wade through pages and pages of work and force, how energy and its conservation were discovered, bouncing molecules and Brownian movement, and flipping coins and shuffling decks, to say nothing of caloric theory and how Carnot's arguments were snatched from its grips, allowing the concept of entropy to emerge. All of this the Goldsteins tell in clear but stiff and uninspired prose, using simple algebraic equations whenever they deem them necessary. There are even mathematical appendices so readers can brush up on units and logs and exponentials. If, *pace* Stephen Hawking, every equation in a popular science book reduces the audience by a half, then I should be the only one that reads this book.

The story is told historically for the most part, although history isn't quite the right word for it. Perhaps hagiography is closer. We are told the tale of one scientific saint after another in a style that is depressingly familiar from high-school and college science textbooks. In fact, the real surprise about this book is that it has not been packaged as a textbook. All it needs is a few problems and discussion items at the end of each chapter and it would serve admirably in a course titled "Thermodynamics for Poets". (Do any such courses exist?)



Top, the long-eared hedgehog (*Hemiechinus auritus*), common in North America and the Middle East; bottom, the pale or Indian hedgehog (*Paraechinus micropus*). Taken from the scholarly *Hedgehogs* by Nigel Reeve, which contains a wealth of information on this familiar and endearing animal. T&AD Poyser, £20.



The dust jacket promises that "*The Refrigerator and the Universe* will appeal to those with little scientific background, but a curiosity about the concepts of energy and entropy, as well as to scientists who use thermodynamics in their research but would like to broaden their understanding of the subject." I am afraid I disagree at both ends of the scale. There are just too many hundreds of pages of plodding before anything interesting happens. To be sure, one really does have to grasp all the preliminaries before one can master the second law, but to get the attention of either the casual reader or the uninformed scientist, that part of the story has to be told with enough wit and insight to make it appealing in its own right. Instead, this book presents readers not with exciting reading, but with medicine they ought to take because it's good for them. I'm afraid that's not good enough to help to solve the worldwide shortage of people who understand the second law. The Goldsteins have made a noble and stout-hearted attempt, but victory over ignorance will have to await another day, and another book. □

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Carnot (1753–1823).

Deuterium abundance and background radiation temperature in high-redshift primordial clouds

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Measurements of the deuterium abundance in the early Universe provide a sensitive test of the 'standard' Big Bang cosmology. The probable detection of deuterium absorption by a gas cloud between us and a distant quasar suggests an abundance much greater than estimated from observations in the Milky Way, and consistent with the amount of presently observed luminous matter comprising all the baryons in the Universe. The same spectra imply a cosmic background temperature for the early Universe which is consistent with our expectations from standard Big Bang cosmology.

THE primary observational supports of Big Bang cosmology are the spectrum and isotropy of the cosmic microwave background radiation (CMBR)¹⁻⁴ and the measurement of light-element abundances predicted by standard Big Bang nucleosynthesis (SBBN)⁵⁻¹⁰. Given a present-day CMBR temperature, SBBN predicts the composition of material emerging from the first few minutes of the Big Bang as a function of the universal density of baryons. Measurements of the relative primordial abundances of H, D, ³He, ⁴He, and ⁷Li test the consistency of SBBN—whether there is any value of the baryon density for which the theory predicts all of these abundances correctly. They also provide a measurement of the baryon density, $\Omega_b h^2$ (in units where the cosmic critical density $\Omega = 1$ and the Hubble constant $H_0 = 100 h \text{ km s}^{-1} \text{ Mpc}^{-1}$), which can be compared with other data—the density of stars, gas and mass in the Universe.

Of the light elements, only H and ⁴He have been measured outside the Galaxy. ⁷Li has been measured in Galactic metal-poor old halo stars, yielding what is presumed to be a nearly primordial abundance, although this interpretation has been questioned¹⁰. As yet, deuterium and ³He have been observed only within the highly evolved and chemically active disk of our Galaxy. Moreover, because D is the most fragile of isotopes, it is easily destroyed in stars, where some of it is converted into ³He and heavier elements, and only some of these daughter products are returned to the interstellar medium. The primordial abundances of these elements have been estimated by modelling the chemical evolution of the Galaxy, a complex process involving the cycling of interstellar gas through generations of stars¹¹. Therefore, in practice the use of these elements in testing the SBBN model has been limited by the need to use evolutionary corrections to the observed abundances. Here we aim at a precise measurement of the ratio of the abundance of deuterium to hydrogen (D/H) in a chemically unevolved environment, bypassing the need for such corrections. The measurement is also confirmation of the predicted universality of the abundances over great distances—a new test of the cosmological principle. Among the light elements, only the ⁴He/H ratio has previously been measured in more than one galaxy, and no ratios have been measured at cosmological distances.

This measurement also affects the SBBN estimate of Ω_b . The most precisely and reliably known primordial abundance is the helium mass fraction, estimated¹² from seven nearby metal-poor

galaxies to be $Y_p = 0.228 \pm 0.005$. The baryon density implied by this estimate alone is rather low, $\Omega_b h^2 = 0.005$, barely more than the number of baryons we know to be present in luminous stars^{13,14} or high-redshift quasar absorbers¹⁵ ($\Omega_b \approx 2.9 \pm 0.6 \times 10^{-3}$). With such a low value for $\Omega_b h^2$ however, SBBN predicts a very high value for deuterium, $D/H \approx 2 \times 10^{-4}$, ~10 times the value of D/H observed in the interstellar medium. Chemical evolution models predict that with such a high D/H, the presolar nebula should be much more enriched in ³He than allowed by observations of the solar wind¹¹. The best chemical evolution model fits, and the constraint of the (D+³He)/H abundance from Solar System measurements, both suggest a primordial D/H in the range $1.9 \times 10^{-5} < D/H < 6.8 \times 10^{-5}$ (for example, ref. 11).

For this reason, the preferred canonical range of baryon density believed^{9,11} to yield the best consistency with all the light elements is $\Omega_b h^2 \approx 0.010\text{--}0.015$. Although this formally predicts Y_p between 2σ and 3σ (where σ is standard deviation) above the observed value, systematic uncertainties in the Y_p determination may be large enough to overpower the formal errors¹³. If the true value of $\Omega_b h^2$ does lie in this range, most of the baryons are in some dark, as yet unobserved, form.

Truly primordial abundances can be measured by studying the absorption spectra of high-redshift quasars. Light from quasars encounters many foreground gas clouds at various redshifts, leading to absorption lines of a variety of species which provide a sensitive probe of cloud composition. This technique offers an opportunity to discover directly the value of D/H in a variety of very distant and fairly chemically unevolved environments, where much less time has elapsed since the Big Bang and where one can verify that little stellar enrichment has occurred, hence little deuterium destruction. Furthermore, some high-column-density quasar absorption clouds—certain of the so-called 'Lyman limit systems' (LLS), which are optically thick to photoelectric absorption by hydrogen—are nearly ideal sites for measuring D/H accurately¹⁶. The H column density of a cloud can be measured quite precisely from the optical depth at the Lyman continuum limit or from line profile fitting of multiple lines of the series. A simple model of a multicomponent gas cloud, consisting of a series of velocity components, each one characterized by a velocity, a column density and spectral 'b-parameter' ($b \equiv (2kT/m)^{1/2}$ where m is the atomic mass and T the temperature) can thereby be overconstrained by simultaneously fitting many lines of the Lyman series. Deuterium is measured by its isotopically shifted absorption lines, displaced towards the blue

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from the hydrogen lines by an apparent velocity $v = cm_e/2m_p = 82 \text{ km s}^{-1}$ (where m_e and m_p are the masses of the electron and proton, respectively). The much smaller D column density is nevertheless detectable and accurately measured in the lowest-order lines.

This important programme has been attempted for years by us and others on 4-m class telescopes without notable success, because the faintness of the high-redshift quasars (that is, high enough redshift to see the Lyman series from the ground) makes high signal-to-noise spectroscopy too difficult at the required resolution— $\sim 10 \text{ km s}^{-1}$ —to constrain the cloud model reliably. In addition, a certain amount of good fortune is required to locate suitably clean absorbers where the deuterium line is not contaminated by other intervening hydrogen lines.

We have now finally obtained data of the required quality, using the recently introduced high spectral resolution spectrograph on the Keck 10-m telescope (Mauna Kea, Hawaii), and report here the result of the first measurements, based on one night of data. We find absorption consistent with a detection $D/H \approx (1.9\text{--}2.5) \times 10^{-4}$ in a chemically unevolved cloud in the line of sight to the quasar Q0014+813; because in any single instance we cannot rule out the possibility of a chance H contamination at exactly the D velocity offset, this result should be considered as an upper limit until further observations of other systems are made. As more measurements are made, we would expect to see a consistent minimum value of D/H emerging as the true, primordial value.

The same spectra also allow a test of another fundamental prediction of relativistic cosmology—namely, that the temperature of the cosmic microwave background radiation T_{CMBR} should evolve with redshift, z , as $T_{\text{CMBR}} \propto (1+z)$ —which is a more direct constraint than other arguments¹⁷ against alternative models for the origin of the background radiation. Recent experiments have provided extraordinarily precise confirmation of the isotropy of the cosmic microwave background radiation and of its black-body spectrum; the current value of T_{CMBR} is now extremely accurately measured, at 2.73 K, both directly^{1,2} and from cyanogen excitation in the interstellar medium¹⁸. The value of T_{CMBR} at high z can similarly be measured through fine-structure splitting of atomic carbon lines in high- z quasar absorption line systems^{19,20}. Although attempts have been made to use this technique on C II and C I lines^{20,21}, the results have again been limited by the difficulty of obtaining very high signal-to-noise spectra on these very faint ($V=16\text{--}17$, where V is visual magnitude) quasars. We present below such a measurement of C II 1,334 Å in the Lyman limit absorber in quasar Q0636+68, which places a new tighter limit of 13.5 K on T_{CMBR} at $z=2.9092$, corresponding to a redshifted value $T_{\text{CMBR}}/(1+z) < 3.5 \text{ K}$ measured at wavelength $611/(1+z) \mu\text{m}$.

Abundance measurements

Observations of the two quasars, Q0636+68 ($V=16.5$) and Q0014+813 ($V=16.9$), were made on 11 November 1993 with the Keck 10-m telescope, as four 40-min exposures and one 50-min exposure for Q0636+68 (3.5 h total), and six 40-min exposures for Q0014+813 (4.0 h total). A $1.2'' \times 3.8''$ slit was used. Each observation covered the range 3,500–5,900 Å at a resolution $R=36,000$ and, at the longer wavelengths of interest here, sky- and read- noise and detector dark current were negligible, so that the signal-to-noise ratio was determined only by source counts.

The two quasars have strong LLS, at $z=2.909$ in Q0636, and $z=2.813$ in Q0014 (ref. 22). Both are metal-line systems and have very complex structure, stretching over several hundred kilometres per second in velocity. In particular, there is contamination by high-negative-velocity hydrogen at the expected position of deuterium, making measurement of D/H essentially impossible. In this they resemble certain interstellar lines of sight²³ which are too complicated to be useful for D/H measurement.

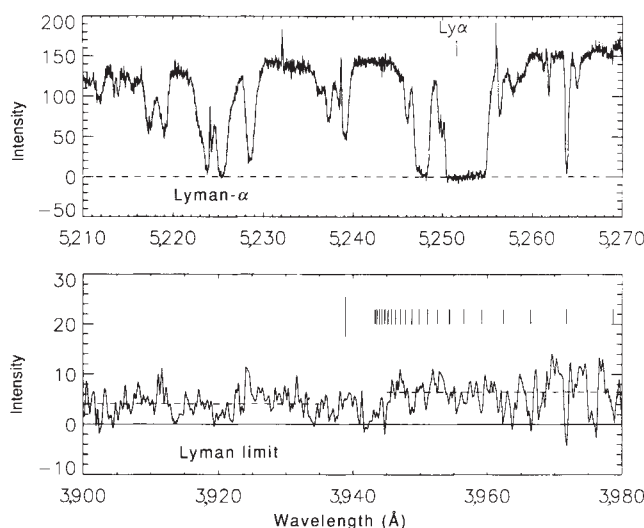


FIG. 1 The region of Lyman- α (5,250 Å; top) and the Lyman break (3,939 Å; bottom) in the $z=3.32$ Lyman limit system (LLS) in the quasar Q0014+813. Six CCD (charge-coupled device) frames were flat-fielded with a quartz lamp exposure, spatially registered and added with a filter to remove cosmic rays; one-dimensional spectra were formed by subtracting a heavily smoothed sky and then summing the spatial points along the slit corresponding to a particular spectral pixel. The spatial summation included all points where the flux exceeded 10% of the maximum on the source. The spectra were precisely wavelength-calibrated using a Th-Ar lamp and approximately flux-calibrated (F_ν) using observations of the white dwarf star Feige 110. The resulting signal-to-noise ratio was ~ 87 per resolution element at Lyman- α and much less (~ 15 per resolution element) at the Lyman break, because of the rapid drop-off in both detector efficiency and the cross-disperser blaze function at and below 4,000 Å. The positions of Ly- α (top) and Lyman 30 and the Lyman break (bottom) are shown as vertical lines. The dashed lines indicate the median values below and above the Lyman break.

Q0014 also has a much weaker LLS at $z=3.3201$ corresponding to the modestly high hydrogen atom column density ($N_H \approx 10^{17} \text{ atoms cm}^{-2}$) metal-poor cloud extensively studied by Chaffee and collaborators^{24,25}. This cloud may contain some elements heavier than helium, as indicated by the possible presence of Si III 1,206 Å and C III 977 Å absorption lines. Although the lines occur at the proper wavelengths, they lie in a region with many Lyman- α absorption lines, and Chaffee *et al.*²⁵ argue that these cannot be attributed to Si and C because the line strengths are not consistent with the expected intensities. Irrespective of this point, the metallicity in the Chaffee cloud is very low. Even in the unlikely case that the cloud were predominantly neutral, the C II and C I line limits ($< 10^{12} \text{ cm}^{-2}$ from our data) would place the metallicity at less than one-fortieth of the solar value, whereas if C III or C IV were the dominant ionization state, the hydrogen would be highly ionized, whether the cloud were photoionized or collisionally ionized^{25,26} and in this case both Chaffee *et al.*²⁵ and Donahue and Shull²⁷ concluded that the metallicity was less than, or about, $10^{-3.5}$ of solar.

Chaffee *et al.* found that the neutral hydrogen in this cloud was well fitted by two components, each with $N_H \approx 5 \times 10^{16} \text{ cm}^{-2}$ and $b \sim 28 \text{ km s}^{-1}$, and with a separation of N_H 110 km s^{-1} . With our higher signal-to-noise, higher resolution data, we have reanalysed the neutral hydrogen model, using the Lyman break at a wavelength of 3,939 Å (Fig. 1) to obtain the combined neutral hydrogen column density. We compared the flux in the 40 Å below the break to that between 3,960 Å and 4,000 Å, and evaluated the ratio of the maximum peaks (0.58), the fifth maximum peaks (0.55), the medians (0.58) and the means (0.60). The maximum peak ratios constitute the best estimate, with the

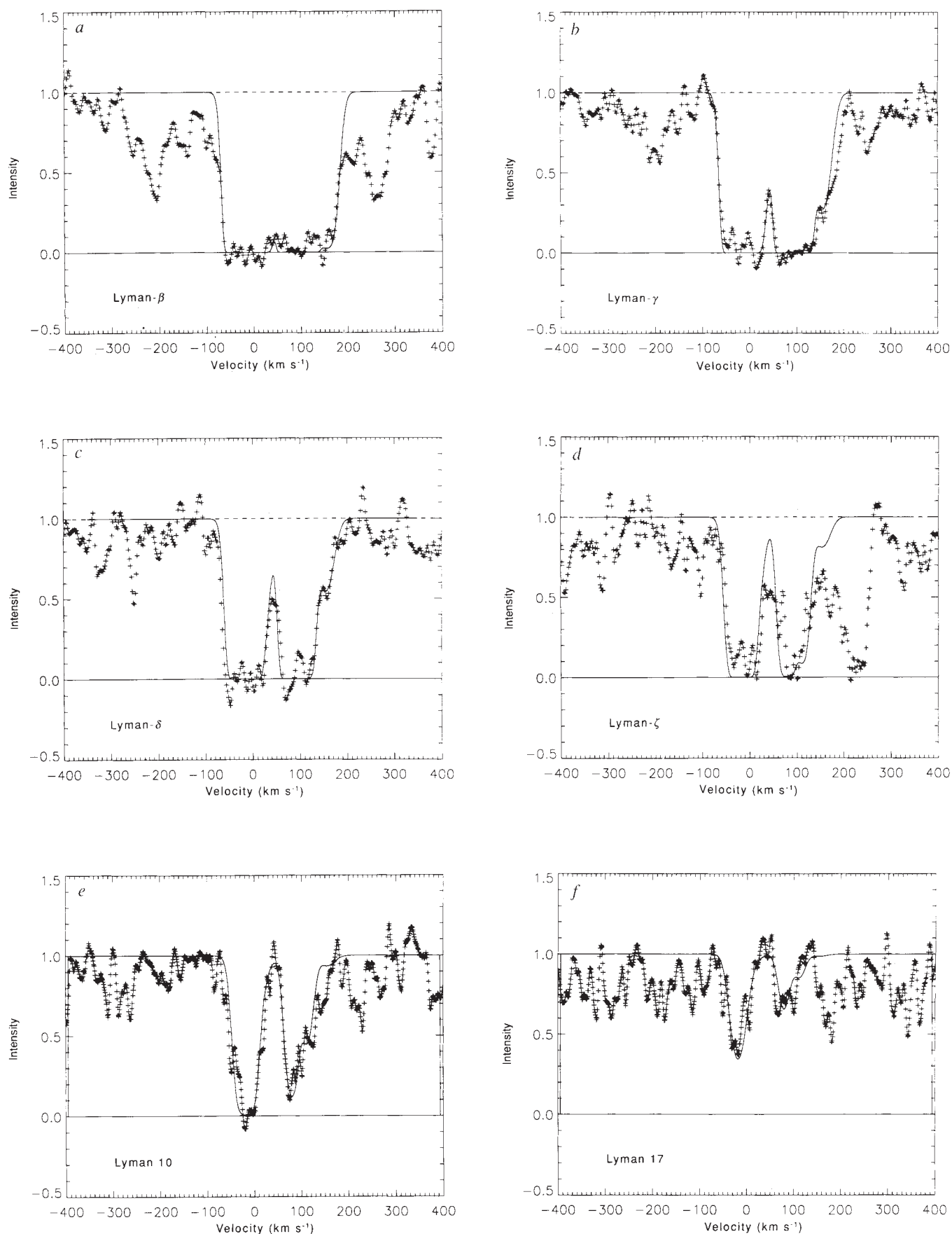


FIG. 2 *a–f*, The fit of the cloud model of Table 1 to Lyman series lines (identified lower left of each panel) of the $z=3.32015$ LLS in Q0014+813. The profile labelled 'Lyman 10' is a median in velocity

space of the Lyman 7 to Lyman 13 profiles, and 'Lyman 17' is a composite of Lyman 14 to Lyman 20. Crosses mark the data values and the solid line the model fit.

mean serving as an upper bound as it is raised by the presence of the Lyman series in the upper wavelength range. Together they give an optical depth $\tau = 0.55 \pm 0.05$, corresponding to $N_{\text{H}} = 9.5 \times 10^{16} \pm 0.8 \times 10^{16} \text{ cm}^{-2}$.

To determine the b -values and relative strengths of the strongest components, which are the key to measuring D/H, it is desirable to work with the unsaturated very-high-order Lyman series lines. The structure of this region is, however, complex, being deep in the Lyman forest, and fitting of individual lines is somewhat subjective (compare Fig. 1). To alleviate this problem we formed the medians in velocity space of the lines Lyman 7 to Lyman 13, and Lyman 14 to Lyman 20 (Fig. 2). This eliminated contaminating lines, leaving only the relatively invariant Lyman lines themselves. In the Lyman 14–20 composite, only the strong component at -17.5 km s^{-1} is clearly seen. The uncertainty in the central velocity is $\sim 5 \text{ km s}^{-1}$. Fitting this with a single cloud, we find a b -value of 21 km s^{-1} with an acceptable range of 18 – 28 km s^{-1} . If we assume that the line approximates Lyman 17, because the oscillator strengths vary only slowly in the high Lyman series, we find $N_{\text{H}} = 5.5 \times 10^{16} \text{ cm}^{-2}$. The primary uncertainty in N_{H} is the placement of the continuum, and the value could range from $8.7 \times 10^{16} \text{ cm}^{-2}$ to $4.0 \times 10^{16} \text{ cm}^{-2}$. The second component at 80 km s^{-1} shows up clearly in the Lyman 7–13 composite. This component is best fitted with two clouds, each with $b = 16 \text{ km s}^{-1}$ and velocities of 80 km s^{-1} and 113 km s^{-1} . The cloud column densities are $N_{\text{H}} = 1.5 \times 10^{16} \text{ cm}^{-2}$ and $5.5 \times 10^{15} \text{ cm}^{-2}$ if we adopt a fit with the oscillator strength of Lyman 10. As we move up the Lyman series, weaker components with $N_{\text{H}} \sim 10^{15} \text{ cm}^{-2}$ are seen, also with b -values around 21 km s^{-1} . The final cloud model is summarized in Table 1 and the fit to the Lyman series is shown in Fig. 2. The total column density of the model fit is $7.9 \times 10^{16} \text{ cm}^{-2}$, which is slightly lower than the value we have estimated from the Lyman break. This could reflect the uncertainty in the strongest component caused by the continuum fit, or possibly some additional material not well described by static thermal slabs; either way, the near agreement argues against significant systematic errors in estimating N_{H} . For the purposes of estimating D/H, we shall assume that cloud 1 has N_{H} in the range $(5.5\text{--}7.3) \times 10^{16} \text{ cm}^{-2}$. This is consistent with the range of $N_{\text{H}} = (2.3\text{--}8.7) \times 10^{16} \text{ cm}^{-2}$ found by Chaffee *et al.*²⁴

The Chaffee cloud is very well suited for a determination of the D/H abundance ratio because the high-column-density component lies at the blue end of the complex. In Fig. 3 we show the

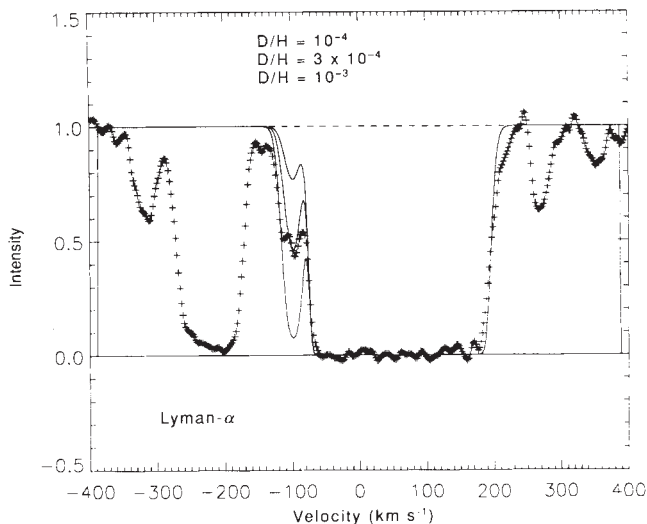


FIG. 3 The prediction of the cloud model of Table 1 for deuterium- α absorption in Q0014+813. Crosses mark data values, and the three solid lines show model fits for $D/H = 10^{-4}$ (top), 3×10^{-4} (middle) and 10^{-3} (bottom).

TABLE 1 Best cloud model ($z = 3.32015$)

v (km s $^{-1}$)	b (km s $^{-1}$)	N (cm $^{-2}$)
−17.5	21.0	5.5×10^{16}
13.5	24.0	1.8×10^{15}
80.0	16.0	1.5×10^{16}
113.0	16.0	5.5×10^{15}
155.0	21.5	6.5×10^{14}

v , Central velocity; b , Doppler parameter; N , column density.

predictions of the cloud model of Table 1 for $D\alpha$ as a function of the value of D/H assuming that $b_{\text{deuterium}} = b_{\text{hydrogen}}/\sqrt{2}$ —that is, thermal broadening. The feature seen in the blue wing of Lyman- α is precisely at the expected position of deuterium and, if interpreted as deuterium, gives a best fit of $D/H = 2.5 \times 10^{-4}$ for $N_{\text{H}} = 5.5 \times 10^{16} \text{ cm}^{-2}$. If the larger value of N_{H} obtained from fitting the break were used, this becomes 1.9×10^{-4} .

Implications for the Big Bang

In the case of a single absorber, we cannot rule out the possibility that an errant hydrogen cloud, with a column density 2.5×10^{-4} that of the main cloud, happens to be floating at the velocity shift where we expect to find deuterium; note in particular that the signal to noise is insufficient to distinguish the narrower thermal broadening expected for D ($b_{\text{deuterium}} = b_{\text{hydrogen}}/\sqrt{2}$). But we can place limits on the likelihood of such a chance interpolation. The redshift of the Chaffee cloud lies close to the redshift of the quasar (3.41) and the number density of Lyman- α forest lines increases steeply with decreasing wavelength in this region. The Ly- α line lies at the red end of echelle order 68, where there are 12 Ly- α forest lines in the observed $75 \text{ \AA}$ which would be strong enough to contaminate the deuterium region, whereas in order 67, which is $80 \text{ \AA}$ to the red, the number has dropped to 4 in $75 \text{ \AA}$. A rate of 12 lines per $75 \text{ \AA}$ would amount to a probability of 3% that a Ly- α line might be observed at random within $\pm 5 \text{ km s}^{-1}$ of the deuterium position. Although this is quite small, until similar abundances are regularly found in other absorbers, it is necessary to regard our measurement as an upper limit on the deuterium abundance. Viewed in this light, the limit is encouragingly close to the values of D/H predicted from Galactic chemical evolution models, and suggests that, as further clouds are studied, we will be able to refine the measurement of D/H to significantly constrain SBBN and chemical evolution models. The quality of the data is sufficient to detect even much lower abundances, so that a small statistical survey of similar absorbers should remove the current ambiguity due to possible contamination.

It is also possible that this might prove to be the best measurement to date of the primordial D/H, and as such it is interesting to examine its potential consequences for cosmological theory. A value of D/H as high as 2.5×10^{-4} is acceptable within SBBN, is just consistent with ^7Li measurements¹⁰, and is even favoured by ^4He (ref. 28). It has the consequence of reducing Ω_b , a factor of three below its canonical value, to $0.005h^{-2}$ (refs 9, 10), which roughly agrees^{13–15} with the inventory of luminous and gaseous matter in the Universe. Because evolution after the Big Bang is a net destroyer of deuterium, it is not surprising to find relatively high D/H in a relatively unevolved primordial cloud.

There are however difficulties reconciling such a high value of D/H with the much lower values found in the interstellar medium²⁹ (of the order of 10^{-5}), and especially with the Solar System abundance of ^3He . Although ^3He can be both created and destroyed in different types of stars, models including a normal mix of stars predict¹¹ that Galactic chemical evolution leads to at best only a slight decrease with time in the sum $(D + ^3\text{He})/H$, so that the $^3\text{He}/^4\text{He}$ ratio measured locally in the solar wind seems to imply a low presolar value of $(D + ^3\text{He})/H \approx 4 \times 10^{-5}$, contradicting our D/H measurement. It is of

course possible that the chemical evolution models are inadequate; for example, it could be that the presolar nebula at the time of its formation contained abundance atypical of the Galaxy. (The possibility of such spatial fluctuations in $^3\text{He}/\text{H}$ is suggested by interstellar measurements of $^3\text{He}/\text{H}$ using hyperfine transitions³⁰, which show a range of values for $^3\text{He}/\text{H}$ from $\leq 1 \times 10^{-5}$ to 15×10^{-5} .) These uncertainties make the use of local ^3He observations ambiguous for cosmological arguments, and motivate further measurements of D/H at high redshift, which should give a much cleaner test of SBBN.

Demonstration of a low value of Ω_b would have a wide impact on other aspects of cosmological theory. For example, many models of galaxy formation predict that the baryon mass fraction in galaxy clusters ought to be Ω_b , and a low value of Ω_b exacerbates their conflict with the observed high baryon fraction ($>0.05h^{-3/2}$) of the Coma cluster³¹. A low baryon density would also conflict with massive spiral galaxy halos being made primarily of baryonic objects, a prediction which can be tested by gravitational microlensing experiments^{32,33}. If we are indeed seeing almost all of the baryons already, dynamical evidence points even more strongly to halos made of non-baryonic dark matter.

Cosmic background temperature

The evolution of the microwave background temperature with redshift is a little-doubted prediction of relativistic cosmology which is nevertheless worth testing precisely. For example, this test complements other arguments^{17,34} constraining alternatives to the Big Bang origin of the background radiation. At the redshifts of our absorbers, we can observe transitions from the ground state of singly ionized atomic carbon C II , as well as from the fine structure split state C II^* which lies 64 cm^{-1} above the ground state. At these redshifts, the frequency difference is only about three times the peak frequency of the CMBR, so C II^* is predicted to have a non-negligible population just due to the presence of the microwave background. We measure the relative populations of C II and C II^* from the strengths of ultraviolet absorption lines from the two states.

We have observed the C II line at $1,334.532 \text{ \AA}$ corresponding to the LLS at $z=2.909$ found by Sargent *et al.*²² in the quasar Q0636+68. The C I multiplets are too weak in this system to be useful for this test while N II lies within the Lyman forest. As Fig. 4 shows, the C II line consists of three strong, and a number of weaker, components stretching over more than $\pm 100 \text{ km s}^{-1}$, the strongest of which has a redshift, $z=2.9034$ which we have chosen to be the zero velocity. Comparison with other lines such as Si II leaves no doubt that all the components seen are C II lines, and confirms the accuracy of the wavelength scale.

Profile-fitting the three strongest components gives a column density, $N_{(\text{CII})} = 4.6 \times 10^{14} \text{ cm}^{-2}$. Considerable additional material could be hidden in strongly saturated components, but this is the minimum value needed to give an accurate fit. By contrast, we do not see the line at $1,335.708 \text{ \AA}$ from the excited fine-structure level C II^* . We have determined the noise level in this region empirically by fitting the column densities at random positions at neighbouring ('wrong') wavelengths and find that the equivalent width of C II^* for all three main components is $0.4 \text{ m\AA} \pm 0.6 \text{ m\AA}$, corresponding to a 2σ upper limit of 10^{12} cm^{-2} in this line. The relative populations of the two states then imply a 2σ limit, $T_{\text{CMBR}} < 13.5 \text{ K}$, which should be compared with the prediction of 10.66 K at this redshift; or, representing the temperature as $T_{\text{CMBR}} = T_0(1+z)^\alpha$, we find that the 2σ upper limit on α is 1.15. This is a firm upper bound: saturation effects could only raise the column density of the C II (and so lower the temperature limit) as would any additional excitation of the C II^* line, other than the CMBR. This is the tightest limit to date on T_{CMBR} at high redshift; the previous best limit of Meyer *et al.*²¹ gave $T_{\text{CMBR}} < 16 \text{ K}$ at $z=1.776$, or $\alpha < 1.73$.

It seems that the fine-structure lines will provide a sensitive new test of relativistic cosmology. The extremely weak excitation

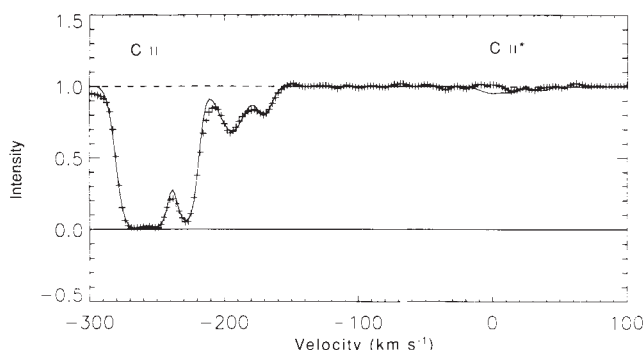


FIG. 4 C II $1,334.532 \text{ \AA}$ and C II^* $1,335.708 \text{ \AA}$ absorption in the $z=2.9034$ LLS in Q0636+68. Crosses show the data values and the positions of C II and C II^* are labelled. The solid line shows a model in which the column density of C II^* is 1% of C II , to show where C II^* would appear if it were present. The rest equivalent width of the three strongest C II components is $248 \text{ m\AA}$, corresponding to a minimum column density $N_{(\text{CII})} = 1.5 \times 10^{14} \text{ cm}^{-2}$. More precisely, profile-fitting gives $N_{(\text{CII})} = 4.6 \times 10^{14} \text{ cm}^{-2}$. The equivalent width of C II^* for all three main components is $\leq 0.4 \text{ m\AA} \pm 0.6 \text{ m\AA}$, corresponding to a 2σ upper limit of 10^{12} cm^{-2} in this line.

of the fine structure levels in this absorber suggests that the population of C II^* is dominated just by the cosmic background, so that we are likely to be able to actually determine T_{CMBR} with improved observations. Because the dependence of equivalent width on temperature is only logarithmic, rather precise measurements of the temperature should be obtainable. In the present case, an increase in signal-to-noise ratio of a factor of 5 (less would be needed at higher redshift) must result in a significant detection of C II^* if the relativistic prediction is correct. Note that systems at different redshifts yield measurements of the background at various present-day frequencies $64/(1+z) \text{ cm}^{-1}$, and so also constrain the shape of the spectrum. Because this technique for measuring the temperature always yields an upper limit, a single well-constrained measurement lower than predicted would have catastrophic repercussions for standard Big Bang theory, whereas a firm measurement of the temperature at the expected level would rule out other possible explanations for the background radiation. $\square$

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- Mather, J. C. *et al. Astrophys. J.* **354**, L37–L40 (1990).
- Mather, J. C. *et al. Astrophys. J.* **420**, 439–444 (1994).
- Smoot, G. F. *et al. Astrophys. J.* **396**, L1–L5 (1992).
- White, M., Scott, D. & Silk, J. A. *Rev. Ast. Astrophys.* (in the press).
- Peebles, P. J. E. *Astrophys. J.* **146**, 542–552 (1966).
- Wagoner, R., Fowler, W. & Hoyle, F. *Astrophys. J.* **148**, 3–49 (1967).
- Reeves, H., Audouze, J., Fowler, W. & Schramm, D. N. *Astrophys. J.* **179**, 909–930 (1973).
- Epstein, R., Lattimer, J. & Schramm, D. N. *Nature* **263**, 198–202 (1976).
- Walker, T. P., Steigman, G., Schramm, D. N., Olive, K. A. & Kang, H. S. *Astrophys. J.* **376**, 51–69 (1991).
- Smith, M. S., Kawano, L. H. & Malaney, R. A. *Astrophys. J. Suppl. Ser.* **85**, 219–247 (1993).
- Steigman, G. & Tosi, M. *Astrophys. J.* **401**, 150–156 (1992).
- Pagel, B. E. J., Simonson, E. A., Terlevich, R. J. & Edmunds, M. G. *Mon. Not. R. astr. Soc.* **255**, 325–345 (1992).
- Pagel, B. E. J. *Phys. Scripta* **T36**, 7–15 (1991).
- Binney, J. & Tremaine, S. *Galactic Dynamics* (Princeton Univ. Press, 1987).
- Wolfe, A. M. *Ann. N.Y. Acad. Sci.* **688**, 281–296 (1993).
- Webb, J. K., Carswell, R. F., Irwin, M. J. & Penston, M. V. *Mon. Not. R. astr. Soc.* **250**, 657–665 (1991).
- Peebles, P. J. E., Schramm, D. N., Turner, E. I. & Kron, R. G. *Nature* **352**, 769–776 (1991).
- Roth, K. C., Meyer, D. M. & Hawkins, I. *Astrophys. J.* **413**, L67–L71 (1993).
- Bahcall, J. N. & Wolf, R. A. *Astrophys. J.* **152**, 701–729 (1968).
- Bahcall, J. N., Joss, P. C. & Lynds, R. *Astrophys. J.* **182**, L95–L98 (1973).
- Meyer, D. M., Black, J. H., Chaffee, F. H., Foltz, C. & York, D. G. *Astrophys. J.* **308**, L37–L41 (1986).
- Sargent, W. L. W., Steidel, C. C. & Boksenberg, A. *Astrophys. J. Suppl. Ser.* **69**, 703–761 (1989).
- Cowie, L. L., Laurent, C., Vidal-Madjar, A. & York, D. G. *Astrophys. J.* **229**, L81–L85 (1979).
- Chaffee, F. H., Foltz, C. B., Röser, H.-J., Weymann, R. J. & Latham, D. W. *Astrophys. J.* **292**, 362–370 (1985).
- Chaffee, F. H., Foltz, C. B., Bechtold, J. & Weymann, R. J. *Astrophys. J.* **301**, 116–123 (1986).

26. Shapiro, P. R. & Moore, R. T. *Astrophys. J.* **207**, 460–483 (1976).
 27. Donahue, M. & Shull, J. M. *Astrophys. J.* **383**, 511–523 (1991).
 28. Vangioni-Flam, E. & Audouze, J. *Astr. Astrophys.* **193**, 81–86 (1988).
 29. Linsky, J. L. et al. *Astrophys. J.* **402**, 694–709 (1993).
 30. Bania, T. M., Rood, R. T. & Wilson, T. L. *Astrophys. J.* **323**, 30–43 (1987).
 31. White, S. D. M., Navarro, J. F., Evvard, A. E. & Frenk, C. S. *Nature* **366**, 429–433 (1993).
 32. Alcock, C. et al. *Nature* **365**, 621–623 (1993).

33. Aubourg, E. et al. **365**, 623–625 (1993).
 34. Wright, E. L. et al. *Astrophys. J.* **420**, 450–456 (1994).

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A diverse new primate fauna from middle Eocene fissure-fillings in southeastern China

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We report the discovery of a fauna of primates from Eocene (~45 Myr) deposits in China having a diversity greater than in European and North American localities of similar antiquity. From the many forms that will illuminate questions of primate phylogeny comes evidence for a basal radiation of primitive simians.

AT present, the fossil record of early primate evolution is strongly biased geographically, with the stratigraphically dense and intensively studied samples of Eocene primates from North America and Europe standing in sharp contrast to the paucity of similar data for Africa and Asia^{1,2}. For example, only eight species of Eocene primates have been described from Asia^{3–10}, and most of these are represented by fragmentary or unique fossils. As a result, the phylogenetic affinities of Eocene Asian primates are controversial^{11–14}, and little is known about how this Asian record relates to those of other continents¹⁵.

Since January 1992 we have worked on richly fossiliferous fissure-fillings located near the village of Shanghuang, in southern Jiangsu Province in the People's Republic of China (PRC) (Fig. 1). These are the first such fillings of Eocene age to be discovered in Asia. The fissures have yielded a large and varied mammalian fauna¹⁶, of which the diversity of Eocene primates is greater than that of the rest of Asia combined.

Like many Eocene localities in North America and Europe, the primate fauna from the Shanghuang fissures includes both adapiforms and omomyids. But the fillings have also yielded distinctive fossil primates that are not found elsewhere: these include the first Eocene tarsiers and fossils that we interpret as basal simians (anthropoids or 'higher primates'). This co-occurrence of adapiforms, omomyids, tarsiers and basal simians is unique, and provides fresh insight into primate phylogeny in general and the biogeographic role of Asia during early phases of primate evolution in particular.

Age and geological setting

The Shanghuang fissure-fillings were formed as middle Eocene karstic infillings into surrounding Triassic carbonates. So far, four fossiliferous fissure-fillings (IVPP locality 93006 A–D) of Eocene age have been sampled. All are exposed in an active commercial quarry near Shanghuang, where the Triassic carbonates are being mined for cement production. Available evidence indicates that the fissure-fillings span only a short interval

(probably 1–2 Myr) of middle Eocene time, with the fauna from fissure D being slightly older than the others.

Biostratigraphically, the mammalian faunas from the fissures appear to represent the Irindmanhan and early Sharamurunion Land Mammal Ages (LMAs), on the basis of taxa such as the lagomorph *Lushilagus lohoensis*¹⁶, the carnivore *Miacis lushiensis*¹⁶, the brontothere *Microtitan*, and the cricetid rodent *Pappocricetodon*. Unfortunately, direct radiometric dating of middle Eocene LMAs in Asia has not yet proven possible. However, the Irindmanhan LMA can be correlated with the Bridgerian and early Uintan LMAs of North America on the basis of a major episode of intercontinental mammalian dispersal¹⁷. In North America the Bridgerian/Uintan boundary occurs at about 46 Myr¹⁸. As a rough appraisal based on these considerations, we estimate the age of the Shanghuang fissures to be about 45 Myr.

Shanghuang adapiforms

Adapiforms¹ are lemur-like early Cenozoic primates that seem to be closely related to living strepsirrhines¹⁹. At least two species are known from the Shanghuang fissures (Fig. 2), where they are most abundant in fissure D.

Aside from several isolated teeth from the Kuldana Formation of Pakistan⁹, the Shanghuang adapiforms comprise the only unequivocal evidence for adapiforms in Asia during the Eocene.

A relatively primitive adapiform is represented by isolated teeth from fissure D. Cheek teeth of this species resemble those of *Europolemur*, an adapiform from the middle Eocene of Europe^{1,20}. In particular, upper molars from Shanghuang resemble those of *E. koenigswaldi* from Messel, Germany²⁰, in that they lack a hypocone. However, absence of a hypocone in these two species is a shared primitive feature, and thus does not reflect a special relationship between them. The Shanghuang species is smaller than any previously named species of *Europolemur*, but further specimens will be required to ascertain whether it represents the first Asian record of this genus.

FIG. 1 Map illustrating geographic location of the Shanghuang fissure-fillings, denoted by an asterisk.

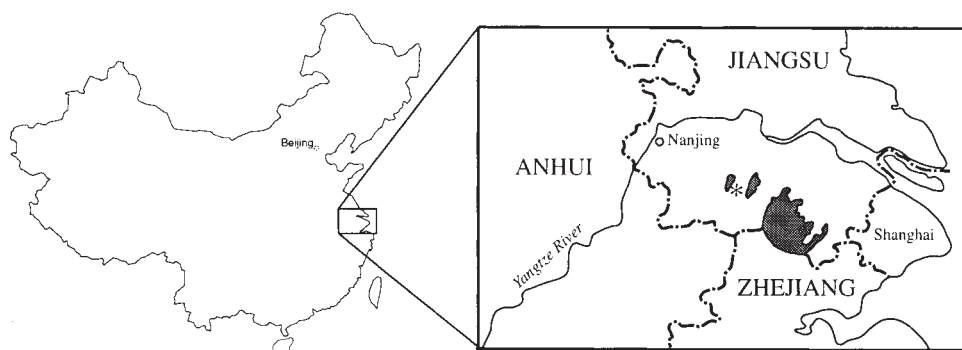
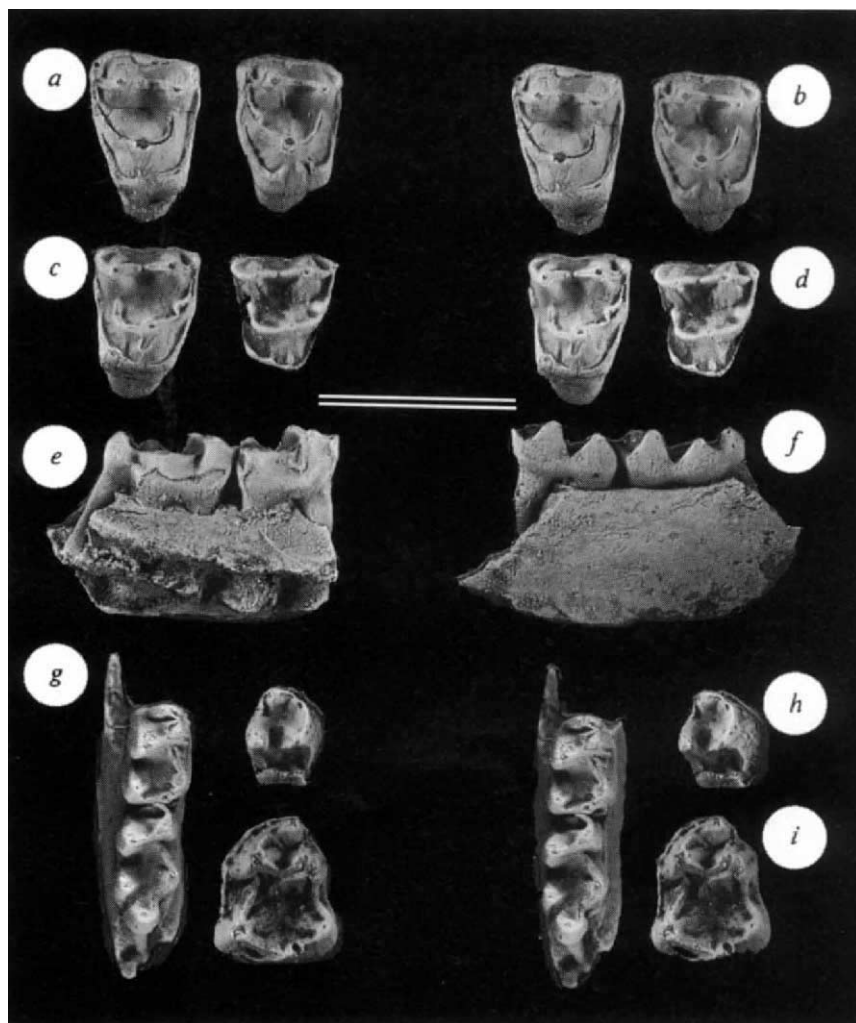


FIG. 2 Adapiform and omomyid primates from the Shanghuang fissure-fillings. *a, b*, *Europoilemur*-like adapiform: IVPP V11019 (*a*), and IVPP V11020 (*b*), each consisting of an isolated left M^1 or M^2 from fissure D, occlusal view. *c–g*, *Adapoides troglodytes*: IVPP V11021 (*c*), isolated right M^1 or M^2 from fissure D, occlusal view; IVPP V11022 (*d*), isolated right deciduous P^4 from fissure D, occlusal view; *e–g*, IVPP V11023, right mandible fragment with M_{2-3} from fissure B, holotype of *Adapoides troglodytes*, in buccal (*e*), lingual (*f*) and occlusal (*g*) views. *h, i*, *Macrotarsius macrorhysis*: IVPP V11025 (*h*), isolated right P_4 from fissure D, holotype of *Macrotarsius macrorhysis*, occlusal view; IVPP V11024 (*i*), isolated left M_1 from fissure D, occlusal view. Occlusal views are stereopairs. Scale bar, 5 mm.



A second adapiform is represented by more nearly complete fossils, which permit its description here.

Order Primates Linnaeus, 1758
Suborder Strepsirhini Geoffroy, 1812
Family Adapidae Trouessart, 1879
Subtribe Adapina Trouessart, 1879
Adapoides troglodytes, new genus and species

Holotype. IVPP V11023, right mandibular fragment preserving M_{2-3} (Fig. 2).

Type locality. IVPP 93006, fissure B.

Known distribution. Middle Eocene of Jiangsu Province, PRC.

Diagnosis. Smaller than *Adapis*, *Cryptadapis* and *Leptadapis*.

Lower molars relatively longer and narrower than in *Leptadapis*. Lower molars without metastylids, in contrast to *Cryptadapis*, *A. parisiensis* and *L. magnus*. Lower molars with high, continuous crest between protoconid and metaconid and deep talonid notch, in contrast to *Microadapis* and *Leptadapis*.

Etymology. From the generic name *Adapis* + Greek suffix *-oides* (like). Trivial name from Greek *troglodytes* (inhabitant of caves).

Description. *Adapoides troglodytes* is a small adapinan, with lower molars (M_2L : 2.65 mm, M_2W : 1.90 mm; M_3L : 3.55 mm, M_3W : 1.80 mm) similar in size to those of *Microadapis sciureus*. The lower molars are primitive in lacking metastylids but derived in having a deep talonid notch and a strong crest uniting proto-

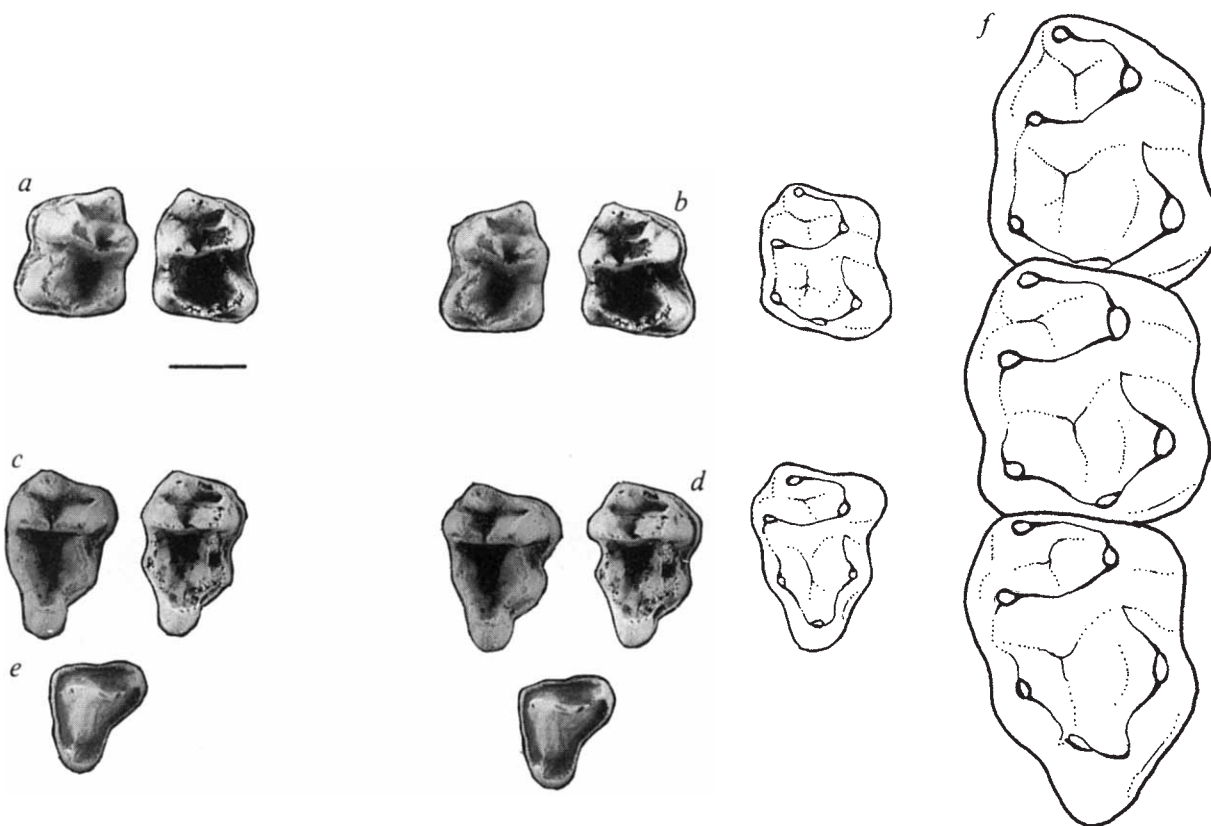


FIG. 3 a–e, *Tarsius eocaenus* from the Shanghuang fissure-fillings: IVPP V11026, isolated left M_1 from fissure A, occlusal view (a); IVPP V11030, isolated right M_1 from fissure C, holotype of *Tarsius eocaenus*, occlusal view (b); IVPP V11027, isolated right M_3 from fissure A, occlusal view (c); IVPP V11031, isolated right M_3 from fissure C, occlusal view (d);

IVPP V11029, isolated left P^3 from fissure C, occlusal view (e). All views are stereopairs; scale equals 1 mm. (f), Dental morphology of *Tarsius eocaenus* (IVPP V11030 and IVPP V11027) compared with that of living *Tarsius bancanus* (USNM 300917), drawn to same scale.

conid and metaconid. Referred upper molars show most of the derived features typical of adapinans (buccal crests form a single mesiodistally oriented shearing surface, metaconule weak or absent, occlusal outline of crown mesiodistally longer and transversely narrower than in *Europolemur* and its close relatives), but primitively lack well-developed hypocones.

Discovery of the Shanghuang adapiforms illuminates two abiding questions about adapiform evolution and biogeography. First, the controversial adapiform *Mahgarita stevensi*, known only from the Duchesnean (late Eocene) of western Texas^{21–23}, has been suggested to be an immigrant from Asia²⁴. Previously, however, there were no Asian adapiforms of suitable morphology to represent a close relative of *Mahgarita*, the affinities of which lie near *Europolemur*^{1,21}. Discovery of a *Europolemur*-like species at Shanghuang provides the first direct evidence that adapiforms of suitable morphology to represent the ancestral stock for *Mahgarita* inhabited Asia during the Eocene, thus corroborating the hypothesis of an Asian origin for this enigmatic genus.

Second, Shanghuang adapiforms also shed light on the phylogeny and biogeography of the subtribe Adapina (including *Adapis*, *Leptadapis*, *Cryptadapis*^{1,25} and *Adapoides*). Anatomically, the Adapina are among the best known Eocene primates, being represented by well-preserved skulls, jaws and numerous postcranial elements^{1,26–28}. However, the phylogenetic and geographic origin of the group has been obscure because the fossil record of these animals was previously limited to Europe, where they appeared suddenly at the beginning of the Robiacian LMA (late Eocene)^{20,29}. Discovery of the small, anatomically primitive adapinan *Adapoides troglodytes* in the middle Eocene of China suggests that adapinans immigrated into Europe from Asia rather than Africa, as had been thought²⁰.

The Shanghuang adapiforms show no special resemblances to the enigmatic Sivaladapinae from the Miocene of India, Pakistan and Yunnan Province, PRC^{30,31}.

Shanghuang tarsiliforms

Both omomyid and tarsiid primates have been recovered from Shanghuang (Figs 2, 3). Omomyids are usually considered to be primitive tarsiiforms^{1,15,32}, although this has been disputed². Remains of omomyids are the rarest primate fossils recovered to date from Shanghuang, where only two isolated teeth have been found in fissure D. Despite their rarity, they are of great interest because they represent a new species of *Macrotarsius*, a genus otherwise known only from western North America^{1,33,34}.

Infraorder Tarsiiformes Gregory, 1915
Family Omomyidae Trouessart, 1879
Macrotarsius macrorhysis, new species

Holotype. IVPP V11025, isolated right P_4 (P_4L , 2.65 mm; P_4W , 2.05 mm) (Fig. 2).

Type locality. IVPP 93006, fissure D.

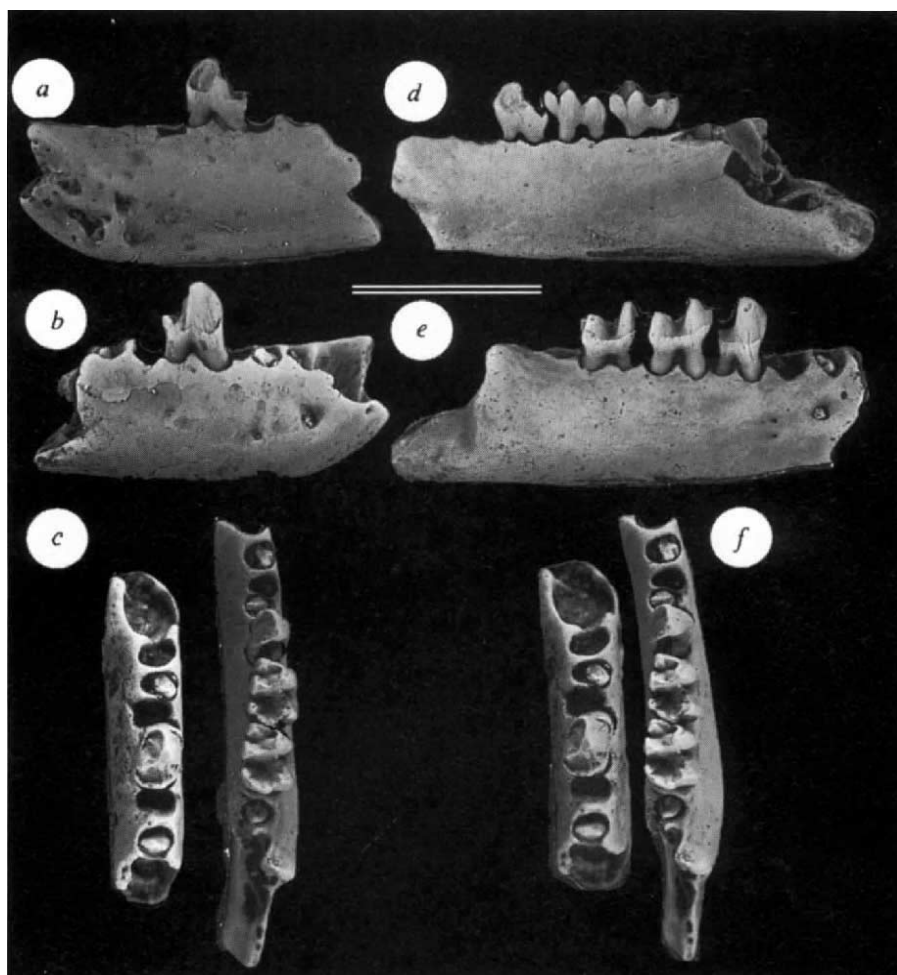
Known distribution. Middle Eocene of Jiangsu Province, PRC.

Diagnosis. P_4 smaller, relatively narrower and with simpler talonid than in other species of *Macrotarsius*. M_1 smaller than in *M. montanus* and *M. siegerti* and without complete ectocingulid, in contrast to *M. roederi*.

Etymology. Trivial name from Greek *makros* (long) + Greek *rhysis* (river), in allusion to the Yangtze River.

Description. *Macrotarsius macrorhysis* closely resembles North American species of *Macrotarsius* in comparable aspects of anatomy: P_4 is mesiodistally short and bears a strongly molarized trigonid, and a well developed crest unites the metaconid and entoconid on M_1 . In terms of size and crown structure, M_1 in

FIG. 4 Eosimiid partial mandibles from the Shanghuang fissure-fillings. *a-c*, IVPP 10592, right mandible fragment with P_4 and alveoli for anterior teeth from fissure A, in *a*, lingual; *b*, labial; and *c*, occlusal views. *d-f*, IVPP 10591, right mandible fragment with P_4-M_2 and alveoli for C_1-P_3 and M_3 from fissure B, holotype of *Eosimias sinensis*, in *d*, lingual; *e*, labial; and *f*, occlusal views. Occlusal views are stereopairs. Scale bar, 5 mm.



M. macrorhysis is practically indistinguishable from that in *M. jepseni*, known from the early Uintan (middle Eocene), Uinta Basin, Utah³³.

Family Tarsiidae Gray, 1825
Tarsius eocaenus, new species

Holotype. IVPP V11030, isolated right M_1 (M_1L , 1.65 mm; M_1W , 1.55 mm) (Fig. 3).

Type locality. IVPP 93006, fissure C.

Known distribution. Middle Eocene of Jiangsu Province, PRC.

Diagnosis. Smaller than other species of *Tarsius*.

Etymology. Trivial name recognizes the Eocene age of this species.

Description. Isolated cheek teeth from fissures A and C attributed to *T. eocaenus* are virtually identical to those of living *Tarsius* in terms of crown structure (Fig. 3). Several aspects of dental anatomy distinguish *T. eocaenus* from omomyids and *Afrotarsius*³⁵. For example, the lower molars of *T. eocaenus* possess shelf-like paraconids situated near the lingual margin of the trigonid; a well developed entocristid uniting the entoconid and metaconid; entoconid positioned near the base of the trigonid, so that the talonid is short on M_1 and the entoconid is mesial to the level of the hypoconid on M_3 (M_2 is unknown); an angular, robust hypoconid; M_3 with a simple, unicusped, distally projecting hypoconulid lobe. Cheek teeth of *T. eocaenus* are uniformly smaller than those of living species of *Tarsius*, including *T. pumilus*³⁶.

The tarsiiform primates from Shanghuang demonstrate for the first time that tarsiids and omomyids were virtually, if not certainly, sympatric during the middle Eocene in southeastern China. Discovery of *Macrotarsius* in Asia confirms previously predictions¹⁵ regarding the feasibility of omomyid dispersal

between North America and Asia during the middle Eocene. More dramatically, *Tarsius eocaenus* nearly triples the palaeontologically documented antiquity of tarsiers via a stratigraphic range extension from the Miocene³⁷ down to the middle Eocene. This brings the fossil record for Tarsiidae into much greater concordance with predictions based on cladistic reconstructions of the phylogenetic position of this group^{2,32,38}.

Basal simians

The most diverse group of primates from the Shanghuang fissures, represented by at least four species, belongs to a further radiation of Eocene primates, interpreted here as basal simians. This radiation has been poorly sampled in the fossil record until now. Although the retention of several primitive features distinguishes these animals from more advanced simian taxa, such a combination of primitive and derived traits is not unexpected in basal simians³⁹⁻⁴². Available evidence does not allow us to determine whether the basal simian taxa sampled to date from Shanghuang constitute a monophyletic or paraphyletic assemblage. Here we describe the basal simian from Shanghuang that is best represented in our current sample.

Suborder Anthroproidea Mivart, 1864
Eosimiidae, new family

Type genus. *Eosimias*, n. gen.

Diagnosis. Differs from Strepsirhini and Tarsiiformes in having the following combination of characters: lower dental formula 2-1-3-3; I_1 small and vertically implanted; C_1 larger than I_1 and P_2 ; P_2 single-rooted; P_3 mesiodistally short and obliquely oriented with respect to tooth row (mesial root more labial than distal root); M_1 with cuspidate paraconid, metaconid widely separated from enlarged protoconid, entoconid situated mesial

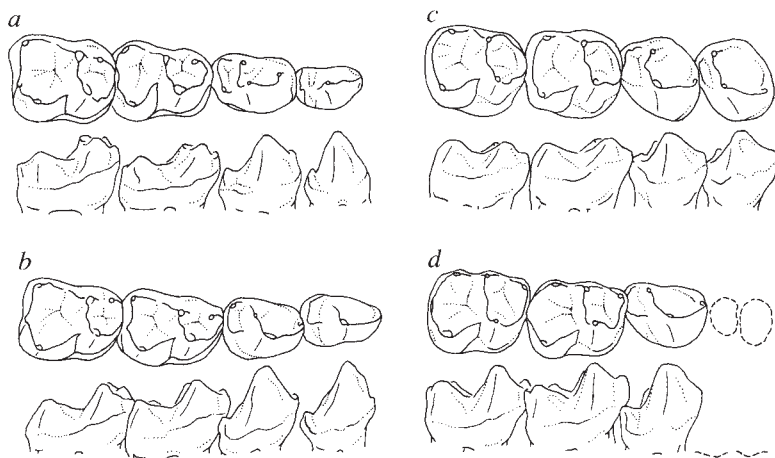


FIG. 5 Comparative schematic drawings of P_3 – M_2 in selected fossil primates (not to same scale). In each pair of drawings, the upper view is occlusal and the lower view is buccal. a, The early Eocene adapiform *Cantius frugivorus* (CM 37448); b, the early Eocene omomyid *Steinius vespertinus* (USGS 25027); c, the middle Miocene simian *Neosaimiri fieldsi* (UCMP 39205); d, the middle Eocene simian *Eosimias sinensis* (IVPP V10591; crowns of P_4 – M_2 only). Note obliquely oriented and mesiodistally short P_{3-4} and similar molar morphology in the simians, in contrast to conditions in *Cantius* and *Steinius*. See text for further discussion.

to level of hypoconid, and distally-projecting hypoconulid.

Differs from other simians (except possibly *Catopithecus*⁴¹, *Proteopithecus*⁴¹, *Serapia*⁴², *Arsinoea*⁴² and *Afrotarsius*^{35,37,40}) in retaining an unfused mandibular symphysis and prominent paraconids on M_{1-2} . Differs from simians except Parapithecidae, *Arsinoea* and possibly *Afrotarsius* in having P_4 metaconid situated well distal and inferior to protoconid, without strong transverse crest uniting these cusps. Differs from Platyrrhini and *Afrotarsius* in having prominent, distally-projecting hypoconulids on M_{1-2} . Differs from Old World simians except *Afrotarsius*, *Amphipithecus* and *Pondaungia* in lacking twinned entoconid and hypoconulid cusps on M_{1-2} .

Eosimias sinensis, new genus and species

Holotype. IVPP V10591, right mandible preserving P_4 – M_2 and roots or alveoli for C_1 , P_{2-3} , and M_3 (Fig. 4).

Type locality. IVPP 93006, fissure B.

Known distribution. Middle Eocene of Jiangsu Province, PRC.

Diagnosis. As for the family (currently monotypic).

Etymology. Greek *eos* (dawn, early) and Latin *simia* (ape). Trivial name refers to geographic provenance of this species.

Description. Aspects of the anterior lower dentition can be inferred from IVPP V10592, a referred specimen from fissure A (Fig. 4). Alveoli for I_{1-2} preserved in this specimen are diminutive. In contrast, the C_1 alveolus is relatively enormous, being much larger than those for I_{1-2} and P_2 . The relative proportions of I_{1-2} in eosimiids are plausibly interpreted as primitive for primates, since they resemble the conditions in other Paleogene simians, adapiforms²⁴ and certain omomyids (e.g., *Washakius*)⁴³. Both IVPP V10591 and V10592 possess an unfused mandibular symphysis (more completely preserved in IVPP V10592). In contrast to adapiforms and omomyids, the symphyseal region of the mandible is dorsoventrally deep rather than strongly procumbent. In this derived feature, *Eosimias* resembles simians.

The crown of P_3 is unknown. Its alveoli, like those of P_4 , are obliquely oriented with respect to the tooth row, the mesial alveolus being situated labial to the distal alveolus. This derived condition does not occur among Eocene adapiforms and omomyids, but is common among Fayum simians (e.g., *Qatrania*)⁴⁴. A similar condition exists in *Amphipithecus* from the Eocene of Burma, a genus widely^{3,12} but not universally¹ considered to be a primitive simian.

P_4 in the holotype is mesiodistally short (1.45 mm) and buccolingually wide (1.25 mm), a derived condition by comparison with such primitive, early Eocene primates as *Cantius* and *Steinius*⁴⁵ (Fig. 5). The moderately exodaenodont (labially bulging) crown is dominated by the protoconid. A weak metaconid occurs distal and well inferior to the protoconid. This position resembles that in parapithecids and *Arsinoea* but contrasts with the condition in other simians, in which the protoconid and metaconid are transversely aligned and united by a strong crest⁴⁰.

Neither a distinct paraconid nor an entoconid is present. The short talonid is open distolingually but is bounded labially by the hypoconid and cristid obliqua. A weak cingulid is discernible labially.

M_1 (length, 1.85 mm; width, 1.40 mm) bears a cuspidate paraconid. Despite this primitive retention, the M_1 trigonid shares derived characters with simians that are not found among Eocene adapiforms and omomyids (Fig. 5). In *Eosimias* the protoconid is enlarged relative to the metaconid and these two cusps are widely separated, as is typical for simians. Among Eocene adapiforms and omomyids these cusps are similar in size and more closely spaced^{1,45}. A premetacristid, also typically absent among Eocene adapiforms and omomyids^{1,45}, unites the metaconid with the base of the paraconid. The talonid, which is wider than the trigonid, is unusual in having the entoconid situated farther mesial than the hypoconid and in possessing a prominent, distally projecting hypoconulid. The buccal cingulid is weak.

M_2 (length, 1.85 mm; width 1.55 mm) is very similar to M_1 , from which it differs in being relatively wider and having a less distally projecting hypoconulid. The paraconid is conspicuous and widely separated mesially from the metaconid. Among Eocene adapiforms and omomyids that retain paraconids on M_2 , this cusp is typically much more connate with the metaconid^{1,45}.

Despite its retention of such primitive characters as diminutive body size (mean estimates based on regressions of body size against M_1 area in living primates⁴⁶ range from 67–137 g), an unfused mandibular symphysis and prominent paraconids on M_{1-2} , *Eosimias* appears to be a primitive simian based on its possession of the following dental synapomorphies: lower dental formula 2-1-3-3; P_2 single-rooted; mesiodistally short, obliquely oriented, and moderately exodaenodont P_{3-4} ; M_{1-2} with enlarged protoconid widely separated from metaconid, premetacristid present, and mesially situated entoconid.

Recent palaeontological discoveries^{41,42} have revealed that the dentitions of early simians retained many primitive features, including lower molar paraconids. These symplesiomorphies complicate attempts to reconstruct phylogenetic relationships among early simians and their relatives, particularly on the basis of dental and jaw anatomy alone. Nevertheless, the combination of characters listed above for *Eosimias* and many early simians is absent in adapiforms and omomyids, suggesting that they are best interpreted as simian synapomorphies. Derived features of *Eosimias* consistently point toward simian affinities, whereas we know of no derived characters for *Eosimias* that suggest an alternative phylogenetic position. Moreover, the specifically simian-like mode of dental reduction and premolar compaction in *Eosimias* differs from that found in certain omomyids (in which this usually occurs in conjunction with hypertrophy of I_1) and adapiforms (none of which evolved obliquely oriented lower premolars as a means of compressing the antemolar dentition)¹.

Within Anthroipoidea, *Eosimias* appears to occupy a very basal phylogenetic position, certainly before the diversification of Parapithecidae, Oligopithecinae, Platyrrhini and Catarrhini (Fig. 6).

Given this position, *Eosimias* yields new insight into the phylogenetic relationships of simians with respect to other primates. One hypothesis holds that early simians evolved from Eocene adapiforms^{23,24,47,48}. The age and anatomy of *Eosimias* cast doubt on this idea. *Eosimias* is smaller than all known adapiforms except the specialized and temporally late *Anchomomys*. Hence, one must postulate an episode of phyletic dwarfing, which is rare among mammals, to derive *Eosimias* from adapiforms other than *Anchomomys*. Moreover, derivation of *Eosimias* from adapiforms such as *Mahgarita* (recently cited²³ as a possible close relative of early simians) would entail at least two implausible character reversals: loss of mandibular symphyseal fusion and neomorphic reacquisition of lower molar paraconids. Rather, *Eosimias* and other recent finds^{32,41,42,48–50} suggest that Anthroipoidea is a very ancient clade that did not evolve from either Eocene adapiforms or omomyids. This is consistent with the monophyly of either Haplorhini (Anthroipoidea + Tarsiiformes) or Prosimii (Strepsirhini + Tarsiiformes), but runs counter to a monophyletic Simiolemuriformes (Strepsirhini + Anthroipoidea). A wide body of evidence supports the monophyly of Haplorhini among living primates^{1,2}, and we prefer this phylogenetic hypothesis at present (Fig. 6).

The discovery of *Eosimias* also bears on hypotheses concerning the biogeographic centre of origin for higher primates. Recent discoveries of Eocene simians in Africa^{41,42,48–50} have emphasized the antiquity of higher primates there, leading some to suggest that simians may have originated on that continent^{40,49,50}. The primitive nature and great age of *Eosimias* suggest, at least, that southeastern Asia was also an important theatre of early simian evolution. The possibility that simians originated in Asia rather than Africa^{11,12} cannot be rejected without further palaeontological evidence from both continents.

If *Eosimias* and related taxa from Shanghuang are basal simians as we suggest, they may help clarify a longstanding controversy concerning the younger Asian primates *Amphipithecus*, *Pondaungia* and *Hoanghoni*. All of these taxa have been compared favourably with early simians^{1,2,47}, and several workers have suggested that some or all of them represent early Asian higher primates^{3,5,11,12,39}. The affinities of these taxa cannot be resolved here, but we note substantive similarities between *Eosimias* and *Amphipithecus*, particularly in the morphology of the lower premolars. These suggest that *Amphipithecus*, and possibly *Pondaungia* and/or *Hoanghoni*, may be younger, more derived

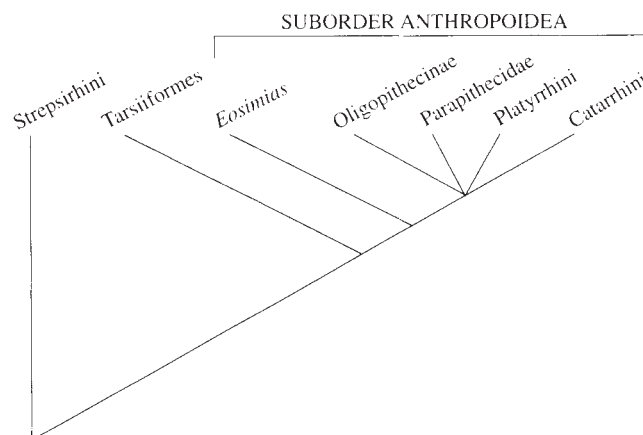


FIG. 6 Cladogram illustrating basal position of *Eosimias* with respect to the evolutionary radiation of simians. Relationships among more advanced simian clades are left unresolved, reflecting the lack of consensus regarding the phylogenetic resolution of this node^{40,41,50}.

members of the basal simian radiation sampled at Shanghuang. If so, the simian affinities of the entire radiation may be better documented by the Shanghuang primates, because of the acquisition of uniquely specialized features through time in the younger *Amphipithecus*, *Pondaungia* and *Hoanghoni*.

Discussion

The Shanghuang primates show surprisingly varied biogeographic affinities, reflecting the complex role played by Asia during early primate evolution. Both Shanghuang adapiforms exhibit clear affinities with European taxa, although the *Europolemur*-like species may be related to North American *Mahgarita* as well. *Macrotarsius* from Shanghuang, like *Asiomomys changbaicus* from Jilin Province, PRC¹⁵, is closely related to North American omomyids. The tarsiid is endemic. The basal simian radiation sampled at Shanghuang, although probably related to younger Asian taxa such as *Amphipithecus* and *Hoanghoni*, may also indicate an ancient (?Palaeocene) biogeographic link with Africa, where early Cenozoic simians are becoming increasingly well known. Although these findings demonstrate that the Eocene primate fauna of Asia interacted with those of other continents through dispersal, many factors underlying the different dispersal patterns of individual taxa remain unknown. □

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- Szalay, F. S. & Delson, E. *Evolutionary History of the Primates* (Academic, New York, 1979).
- Martin, R. D. *Nature* **363**, 223–234 (1993).
- Pilgrim, G. E. *Mem. Geol. Surv. India* **14**, 1–26 (1927).
- Zdarsky, O. *Palaeontol. Sinica* **6**, 1–87 (1930).
- Colbert, E. H. *Am. Mus. Novitates* **951**, 1–18 (1937).
- Chow, M. *Vert. Palaeontol.* **5**, 1–5 (1961).
- Dashzeveg, D. & McKenna, M. C. *Acta Palaeontol. Polonica* **22**, 119–137 (1977).
- Russell, D. E. & Gingerich, P. D. *C. R. Acad. Sci., Paris (Sér. D)* **291**, 621–624 (1980).
- Russell, D. E. & Gingerich, P. D. *C. R. Acad. Sci., Paris (Sér. II)* **304**, 209–214 (1987).
- Wang, B. & Li, C. *Vert. Palaeontol.* **28**, 165–205 (1990).
- Maw, B., Clochon, R. L. & Savage, D. E. *Nature* **282**, 65–67 (1979).
- Clochon, R. L., Savage, D. E., Tint, T. & Maw, B. *Science* **229**, 756–759 (1985).
- Rose, K. D. & Krause, D. W. *J. Mamm.* **65**, 721–726 (1984).
- Gingerich, P. D., Dashzeveg, D. & Russell, D. E. *Geobios* **24**, 637–646 (1991).
- Beard, K. C. & Wang, B. *Am. J. Phys. Anthropol.* **85**, 159–166 (1991).
- Qi, T., Zong, G.-F. & Wang, Y.-Q. *Vert. Palaeontol.* **29**, 59–63 (1991).
- Russell, D. E. & Zhai, R.-J. *Mem. Mus. nat. Hist. nat. (Sér. C)* **52**, 1–488 (1987).
- Prothero, D. R. & Swisher, C. C. in *Eocene-Oligocene Climatic and Biotic Evolution* (eds Prothero, D. R. & Berggren, W. A.) 46–73 (Princeton Univ. Press, Princeton, 1992).
- Beard, K. C., Dagosto, M., Gebro, D. L. & Godinot, M. *Nature* **331**, 712–714 (1988).
- Franzen, J. L. *Cour. Forsch.-Inst. Senckenberg* **91**, 151–187 (1987).
- Wilson, J. A. & Szalay, F. S. *Folia Primatol.* **25**, 294–312 (1976).
- Wilson, J. A. & Stevens, M. S. *Univ. Wyoming Contrib. Geol. Spec. Pap.* **3**, 221–235 (1986).
- Rasmussen, D. T. *Int. J. Primatol.* **11**, 439–469 (1990).
- Gingerich, P. D. in *Evolutionary Biology of the New World Monkeys and Continental Drift* (eds Clochon, R. L. & Chiarelli, A. B.) 123–138 (Plenum, New York, 1980).
- Godinot, M. *C. R. Acad. Sci., Paris (Sér. II)* **299**, 1291–1296 (1984).
- Gingerich, P. D. & Martin, R. D. *Am. J. Phys. Anthropol.* **56**, 235–257 (1981).
- Dagosto, M. *Folia Primatol.* **41**, 49–101 (1983).

- Godinot, M. & Joffroy, F. K. in *Actes du Symposium Paléontologique Georges Cuvier* (eds Buffetaut, E., Mazin, J. M. & Salmon, E.) 221–242 (Le Serpentaire, Montbéliard, 1984).
- Franzen, J. L. & Haubold, H. *Mod. Geol.* **10**, 159–170 (1986).
- Gingerich, P. D. & Sahni, A. *Int. J. Primatol.* **5**, 63–79 (1984).
- Pan, Y. *J. hum. Evol.* **17**, 359–366 (1988).
- Beard, K. C., Krishtalka, L. & Stucky, R. K. *Nature* **349**, 64–67 (1991).
- Krishtalka, L. *Ann. Carnegie Mus.* **47**, 335–360 (1978).
- Kelly, T. S. *Nat. Hist. Mus. Los Angeles County, Contrib. Sci.* **419**, 1–42 (1990).
- Simons, E. L. & Bown, T. M. *Nature* **313**, 475–477 (1985).
- Musser, G. G. & Dagosto, M. *Am. Mus. Novitates* **2867**, 1–53 (1987).
- Ginsburg, L. & Mein, P. C. *R. Acad. Sci., Paris (Sér. II)* **304**, 1213–1215 (1987).
- Norell, M. A. & Novacek, M. J. *Science* **255**, 1690–1693 (1992).
- Delson, E. & Rosenberger, A. L. in *Evolutionary Biology of the New World Monkeys and Continental Drift* (eds Clochon, R. L. & Chiarelli, A. B.) 445–458 (Plenum, New York, 1980).
- Fleagle, J. G. & Kay, R. F. *J. hum. Evol.* **16**, 483–532 (1987).
- Simons, E. L. *Proc. natn. Acad. Sci. U.S.A.* **86**, 9956–9960 (1989).
- Simons, E. L. *Proc. natn. Acad. Sci. U.S.A.* **89**, 10743–10747 (1992).
- Covert, H. H. & Williams, B. A. *J. hum. Evol.* **21**, 463–467 (1991).
- Simons, E. L. & Kay, R. F. *Am. J. Primatol.* **15**, 337–347 (1988).
- Rose, K. D. & Bown, T. M. *Proc. natn. Acad. Sci. U.S.A.* **88**, 98–101 (1991).
- Conroy, G. C. *Int. J. Primatol.* **8**, 115–137 (1987).
- Rasmussen, D. T. & Simons, E. L. *Folia Primatol.* **51**, 182–208 (1988).
- Simons, E. L. *Science* **247**, 1567–1569 (1990).
- de Bonis, L., Jaeger, J.-J., Coiffat, B. & Coiffat, P.-E. *C. R. Acad. Sci., Paris (Sér. II)* **306**, 929–934 (1988).
- Godinot, M. & Mahboubi, M. *Nature* **357**, 324–326 (1992).

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The shape, expansion rate and distance of supernova 1993J from VLBI measurements

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SUPERNOVA 1993J is one of the closest supernovae discovered this century, allowing observations with very high linear resolution. Radio emission from the supernova became visible within days of the first optical peak, and has remained strong, making this an ideal source for very-long-baseline interferometry (VLBI) investigations^{1–7}. Here we report the results of a series of VLBI observations, made from one to three months after the supernova explosion. We find that the supernova is circularly symmetric, which is somewhat surprising in view of suggestions that the progenitor was a member of a binary system^{8,9}, and the asymmetry implied by optical observations¹⁰. The supernova shows no sign of deceleration, and the expansion velocities that we estimate from the VLBI measurements are consistent with the maximum optical line velocities¹¹, suggesting that the radio emission indeed arises from the shock front, where the ejecta are hitting the gas that surrounded the progenitor star. We combine the angular expansion rate determined by the VLBI data with the optically derived expansion speed to estimate a distance to M81 of 4.0 ± 0.6 Mpc, consistent with the value obtained from measurements of Cepheid variables in M81, 3.63 ± 0.34 Mpc (ref. 12).

Our measurements were made with a global VLBI array of between 9 and 15 antennas, observing for up to 18 h at one to four frequencies at each of three epochs (Table 1). We correlated the data from a large subarray of antennas, representing ~30% of all the data, at Haystack Observatory; the full data sets will be correlated with the NRAO very-long-baseline array processor at Socorro, New Mexico, USA.

Iterative self-calibration of the data, imaging and model-fitting were carried out using the California Institute of Technology VLBI package and NRAO's Astronomical Image Processing System. In Fig. 1 we display images at 22 GHz from the first epoch and 8.4 GHz from the second epoch, when the supernova was still rather opaque. These images show a compact brightness distribution with no hint of protrusions⁵ or structure in general beyond the size of the convolving synthesized beam, down to 8% and 1.5%, respectively, of the peak surface brightness. The visibility data (Fig. 2) indicate that the supernova is indeed, at each epoch, smaller than the synthesized beam, but clearly

extended and expanding. We therefore used simple geometric models to determine precise values for the size and the nominal ellipticity of the supernova, starting in each case in our iterative process with a point source.

The estimated model parameters are listed in Table 1; all uncertainties are intended to represent one standard deviation (1σ), with statistical and systematic contributions combined. The quoted angular radii refer to a uniform-disk model and are intermediate between the radii of other reasonable models used in the fit to the same visibilities. The radii would be larger by a scaling factor of $\kappa = 1.12$ had we used an optically thin uniform sphere model, and smaller ($\kappa = 0.88$ to 0.84) had we used an optically thin shell model with a shell thickness less than 20% of the radius¹³. The supernova was still too compact to distinguish between the models. Which model is actually appropriate depends both on the underlying brightness distribution without absorption, and on opacity effects¹³. Because we observed the supernova at frequencies ν near the turnover frequency ν_m (Table 1), the opacities are significant. The effect of those opacities, will, however, depend on where the absorption occurs. In the original circumstellar interaction model^{14,15} (in which the collision of the outer supernova gas with the dense wind from the progenitor star creates a high-energy-density interaction shell), the absorption takes place in the medium surrounding the radio-emitting shell. In this case, the observed brightness distribution is barely affected at our frequencies, for which $\nu \geq 0.5 \nu_m$. If the absorbing medium is, instead, mixed with the emitting material, the observed brightness distribution will be more disk-like than in the optically thin case, regardless of the geometry of the emission region. Our present results are not accurate enough to distinguish between internal and external absorption; after a complete correlation of the data, we may be able to do so.

Is the radio supernova spherically symmetric? Neither the amplitudes and closure phases of our data nor our best-fit elliptical models indicate any significant elongation of the supernova. The nominal position angles are scattered over a large range. We conclude that the radio emission region is circularly symmetric within 5% (1σ). This lack of any significant deviation from circular symmetry may be surprising if one considers recent indications that the progenitor of the supernova had a binary companion^{8,9}, which may have affected the shock front or the density distribution of the circumstellar medium. Our result also contrasts with spectropolarimetric observations of SN1993J, which suggest aspherical geometries for the supernova ejecta with axis ratios of ≤ 0.65 for oblate and ≤ 0.5 for prolate scattering envelopes¹⁰. However, as Trammell *et al.*¹⁰ note, clumpiness of the scattering medium, different scattering models, or a change in the correction for the interstellar optical polarization¹⁴ could account for the spectropolarimetric observations, even in the case of more spherically symmetric supernova ejecta.

How fast does the supernova expand? A weighted least-squares linear fit to the radii of the circular models gives a zero-point of expansion of $March\ 25 \pm 4$ and an expansion velocity $\Theta = 2.86 \pm 0.19 \mu\text{s d}^{-1}$ with a χ^2_ν of 0.77. This date is consistent with the more precise shock-breakout date of $March\ 28.0 \pm 0.1$ obtained from optical data¹⁵. If we constrain the fit to originate at the latter date, we get $\Theta = 2.98 \pm 0.08 \mu\text{s d}^{-1}$ with $\chi^2_\nu = 0.71$ (Fig. 3). A power-law fit, $\Theta(t) \propto t^m$, where t is time, also constrained to originate at the latter date, gives $m = 0.96 \pm 0.07$ with $\chi^2_\nu = 0.79$. The expansion rate is therefore consistent with being uniform.

Is the radio emission linked to the shock front? If one assumes a value for the distance derived from Hubble Space Telescope observations of Cepheids in M81 of $D = 3.63 \pm 0.34$ Mpc (ref. 12), our angular (power-law) expansion rate in mid-April corresponds to a transverse velocity of $19,000 \pm 3,000 \text{ km s}^{-1}$, including a 12% model uncertainty ($\kappa = 1.00 \pm 0.12$) as discussed above. We compared this value with estimates of the radial shock front velocity from spectra provided by the Isaac Newton group

of telescopes on La Palma and in some cases from spectra taken with the David Dunlop Observatory near Toronto¹¹. The maximum expansion velocity of the supernova ejecta, $v_{H\alpha\max}$, say, between 12 and 20 April 1993 was $18,000 \pm 1,000 \text{ km s}^{-1}$, measured with respect to the blue edge of the $H\alpha$ absorption trough (see also refs 10, 18). To translate transverse to radial motions, we assume a velocity isotropy factor of $\mu = 1.00 \pm 0.05$ based on our measurements of the supernova's circular symmetry. The ratio η between the maximum velocities of the radio emission region and the $H\alpha$ gas is then $\eta = 1.06 \pm 0.18$, indeed linking the radio emission region to the shock front. This result is consistent with the prediction of the circumstellar interaction model^{14,15}. In this model, radio synchrotron radiation is pro-

duced by relativistic electrons accelerated at the shock front^{19,20} (see also ref. 21). When the shock front propagates through a circumstellar medium left over from the progenitor of the supernova, and the medium has a density profile $\rho \propto r^{-s}$ with $0 \leq s \leq 2$ and r as the distance from the explosion centre, then $1.1 \leq \eta \leq 1.2$ for a deceleration index $m \approx 0.95$ (refs 14, 15). Previously, only lower bounds on η of 0.65 to 0.75 were determined²² or could be inferred²³ for a supernova, namely SN1987A.

The lack of significant deceleration constrains the density profile $\rho \propto r^{-n}$ of the supernova ejecta in the shock front region. In the circumstellar interaction model $m = (n-3)/(n-s)$. Modelling of the radio lightcurve at several frequencies indicates $s \approx 1.5$ (ref. 24). Our measurement of m then leads to a rather

FIG. 1 *a*, "Clean" map of SN1993J at 22.2 GHz 30 days after shock breakout. The contours are at 8, 12, 16, 20, 30, 40, 60 and 80% of the peak surface brightness. The synthesized beam is shown as a filled circle (at lower left) and has a full-width at half-maximum (FWHM) of 0.26 mas. *b*, "Clean" map at 8.4 GHz 50 days after shock breakout. The contours are at 1.5, 3, 5, 8, 12, 16, 20, 30, 40, 60 and 80% of the peak surface brightness. The beam has a FWHM of 0.61 mas. Note that the larger apparent size of the supernova image in *b* is mostly due to the larger beam. The grey backgrounds indicate peak-to-peak noise fluctuations of 2% (*a*) and <2% (*b*) of the respective peak surface brightness. 1 mas is equivalent to $6 \times 10^{11} \text{ km}$ for a distance of 4 Mpc.

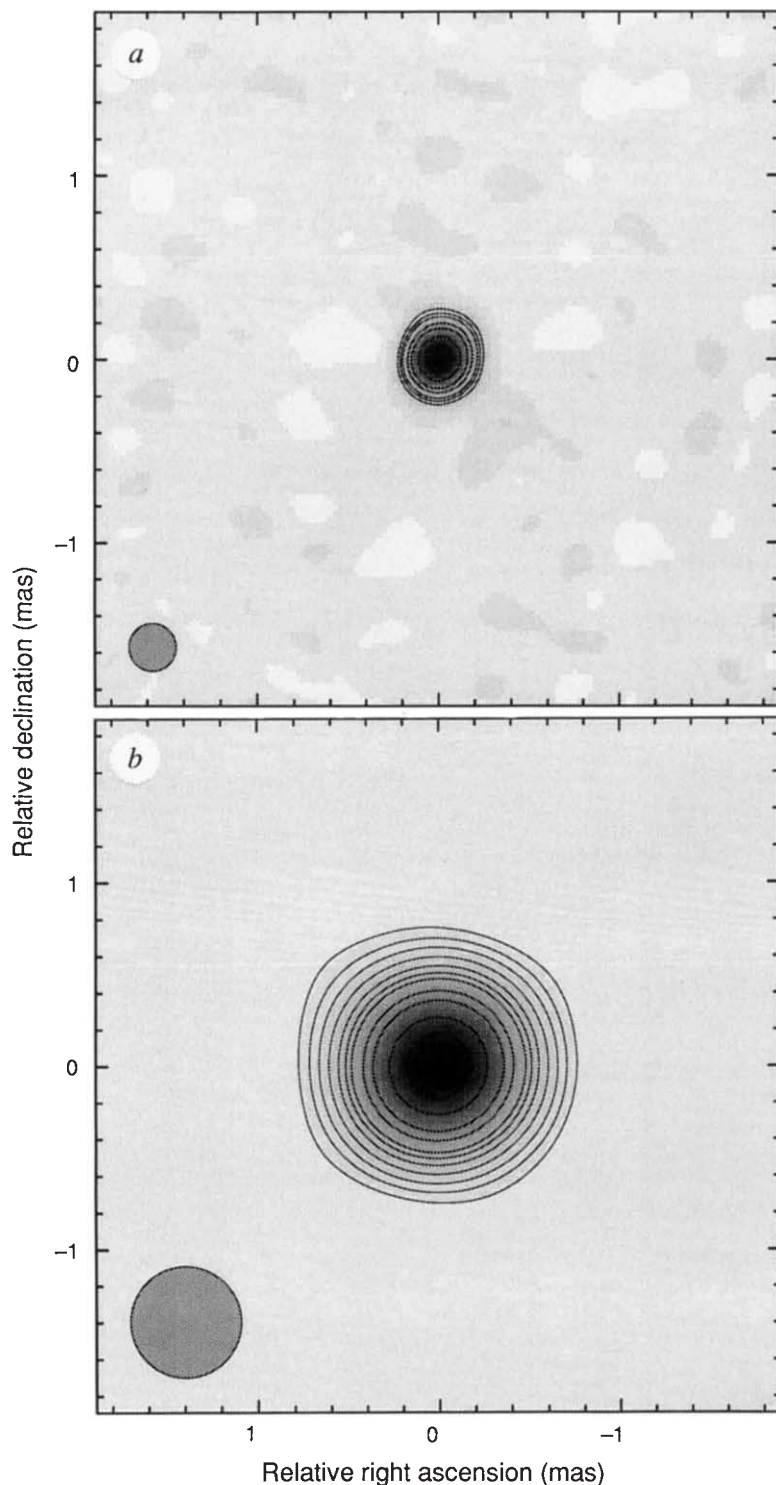


TABLE 1 Description of observations and parameters of uniform-disk models of SN1993J

Session number	Date of observation (UT)	Age* (d)	Observing frequency (GHz)	$\nu/\nu_m^\dagger$	Total flux density ‡ (mJy)	Circular model: radius § (μ s)	Elliptical model: axis ratio $^\S $	Elliptical model: position angle of major axis § (deg)
1	April 27.2	30.2	22.235	1	47.5 $\pm$ 2.4	96 $\pm$ 8	0.78 $\pm$ 0.08	135
2	May 17.2	50.2	22.235	1.5	54.6 $\pm$ 2.8	145 $\pm$ 14	0.80 $\pm$ 0.08	85
			14.867	1	74.4 $\pm$ 4.7	—	—	—
			8.417	0.5	61.9 $\pm$ 3.1	151 $\pm$ 11	0.84 $\pm$ 0.06	105
3	June 27.0	91.0	22.235	2.5	55.6 $\pm$ 5.6	—	—	—
			14.867	2	78.3 $\pm$ 5.9	241 $\pm$ 21	0.92 $\pm$ 0.09	110
			8.417	1	103.3 $\pm$ 5.2	275 $\pm$ 9	0.90 $\pm$ 0.09	45
			4.860	0.5	80.6 $\pm$ 4.1	205 $\pm$ 85	—	—

The observations were made with the following antennas (diameters, affiliations and locations in parentheses): VLA (equivalent diameter 130 m, NRAO, near Socorro, New Mexico, USA); Ef (100 m, MPIfR, Effelsberg, Germany); Ro (70 m, NASA-JPL, Robledo, Spain); Go (70 m, NASA-JPL, Goldstone, California, USA); Ag (46 m, ISTS/York Univ., Algonquin Park, Ontario, Canada); Gb (43 m, NRAO, Greenbank, West Virginia, USA); Mc (32 m, IdR-CNR, Medicina, Italy); Nt (32 m, IdR-CNR, Noto, Italy); Cm (32 m, NRAL, Cambridge, UK); VLBA (25 m, NRAO, 9 out of 10 antennas spread out across the USA). At each station a hydrogen maser frequency standard was used to govern the local oscillator chain and the 'time tagging' of the recorded data. The data were taken with the Mark IIIA and the VLBA VLBI systems³¹ in mode B double speed, generally yielding bandwidths of 56 MHz. Left-hand circular polarization (IEEE convention) was recorded at 22 and 5 GHz and right-hand circular polarization at 15 and 8 GHz.

* The age of the supernova with respect to the estimated data of March 28.0 UT for the shock breakout as deduced from the optical lightcurve¹⁰.

† The ratio of the observing frequency to the turn-over frequency, that is, the frequency at which the spectrum is flat ($\alpha=0$, $S_\nu \propto \nu^\alpha$ where S_ν is the flux density at frequency ν).

‡ The total flux densities were obtained from observations with the VLA which were made simultaneously with, and essentially at the same frequencies as, the VLBI observations during the sessions 1 and 2. The values for the third session were obtained with the VLA less than one day before the start of our VLBI observations at centre frequencies slightly different from those listed, namely at 22.46, 14.94, 8.44 and 4.86 GHz, respectively.

§ For the model fitting we averaged the visibility data into 2-h bins. The data uncertainties were taken either from the number of bits correlated or from the r.m.s scatter of the data within each bin, whichever was larger. They were then added in quadrature to an additional relative uncertainty of 2% for the amplitudes and of 1° for the phases to account for effects which cannot be removed by self-calibration. The χ^2_ν (χ^2 divided by number of degrees of freedom, ν) for each of our model fits was $\chi^2_\nu \leq 1.2$ except in the case of the 4.86 GHz data of the third session, for which $\chi^2_\nu = 1.4$. The uncertainties of a model parameter, x , were derived by off-setting the parameter slightly by Δx from its best-fit value, x_0 , and keeping it fixed at $x_0 + \Delta x$ while adjusting the other parameters and the time-varying gains (typically 50 parameters) of the telescopes in the self-calibration process. This approach automatically takes into account correlations between the model parameters and the antenna gains. The resulting χ^2 was then compared with the best-fit χ^2_0 . The uncertainties of the model parameters refer to increases of χ^2 with respect to χ^2_0 by a factor $(\nu+1)/\nu$ and are 1 standard deviation (1σ) with the statistical uncertainties and most systematic uncertainties included.

$||$ The ratio of the minor to the major axis. As the maximum possible ratio is 1.0, the presence of noise biases the ratio towards smaller values. The expectation value for the axis ratio of an elliptical model used to fit data from a circularly symmetric source with the noise from, for instance, session 1 is ~ 0.9 . The weighted mean of all our axis ratios in the table is 0.85 ± 0.05 , which is within about 5% (1σ) of that expected for a circularly symmetric source.

steep profile with $n \geq 15$. On the other hand, the lack of significant acceleration argues against centrally powered pulsar nebula models^{25,26}. For instance, optical observations of the Crab Nebula give $m = 1.08 \pm 0.01$ (ref. 27) and radio observations give $m = 1.24 \pm 0.15$ (ref. 28), each of the values being somewhat larger than our upper bound.

Because the size and expansion rate of the supernova are frequency-independent within the uncertainties, we combined them with the flux densities obtained during a Very Large Array monitoring programme²⁴ to calculate brightness temperatures, T_b . During the fast flux density rise of the supernova, at each frequency T_b remains $\sim 1 \times 10^{10}$ K within a factor of ~ 2 . Only when the radio lightcurve flattens does T_b decrease. This behaviour is expected for an expanding, optically thick source during the rapid rise, followed by a decline in opacity as the radio flux density reaches its peak (see for example ref. 29).

We can estimate the distance D of the supernova directly, and independently of the Cepheid method, by dividing the linear by the angular expansion rate^{1,2}. More precisely, $D = v_{\text{Hmax}}(t)\eta\mu/\dot{\Theta}(t)\kappa$. As the supernova ejecta are (almost) certainly optically thick for radio radiation so shortly after the explosion, $\eta > 1$; and with our above measurements of the remaining parameters, we get a robust bound of $D > 3.4 \pm 0.5$ Mpc. With $\eta = 1.15 \pm 0.07$, from the circumstellar interaction model, we obtain a fairly direct measurement of the distance to M81 of $D = 4.0 \pm 0.6$ Mpc where the uncertainty combines all significant statistical and systematic errors. Our measurement lies between previous distance estimates, which range from 2.6 ± 0.4 Mpc (ref. 18) to 5.7 Mpc (ref. 30), and is consistent with a value of 4.2 ± 0.6 Mpc (ref. 15) and the above estimate from Cepheid observations¹².

Further multi-frequency VLBI observations of SN1993J continue to be done at regular intervals and may allow us to

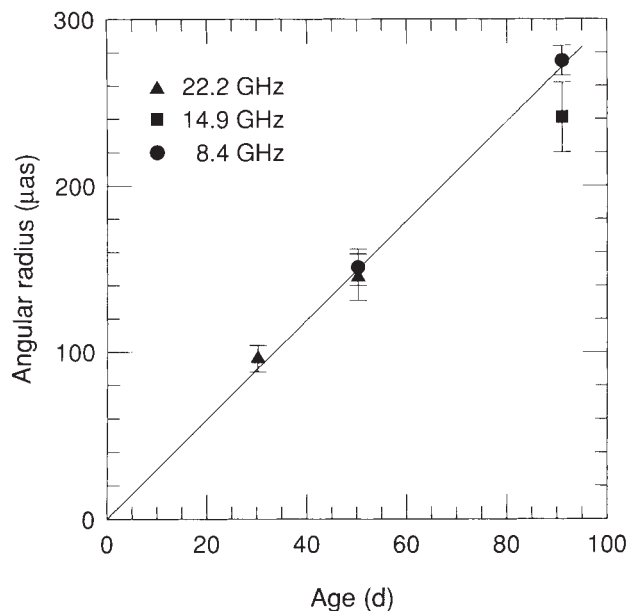


FIG. 3 Angular radius of SN1993J with respect to a circular uniform-disk model of the supernova's brightness distribution, plotted against age measured from 28.0 March 1993 UT. The 4.9 GHz value with its large uncertainty is omitted for clarity. The straight line represents a least-squares linear fit to all data.

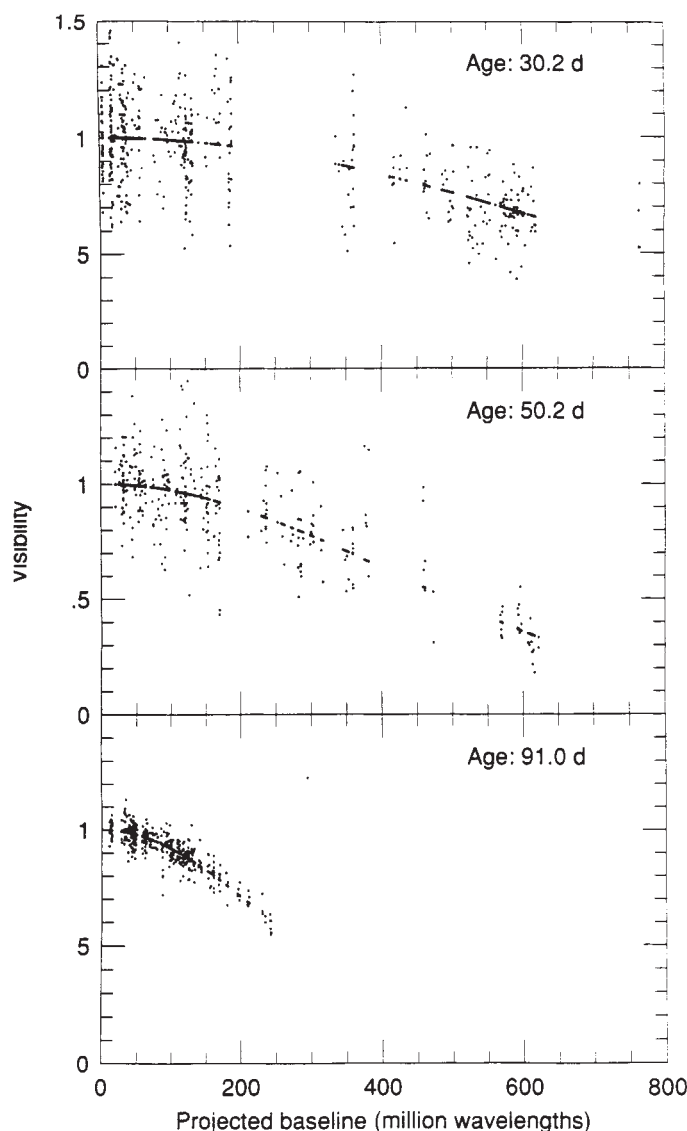


FIG. 2 Measured visibility amplitudes of SN1993J and predictions from the fit of a circular uniform-disk model for three epochs. The data from the first two epochs were taken at 22.2 GHz and from the last epoch at 8.4 GHz. The visibility amplitudes would have been constant along the projected baseline length if the supernova were completely unresolved. The curved slope indicates that the supernova is slightly extended, but not beyond the FWHM of the synthesized beam. The increasingly steeper slope at consecutive epochs clearly shows that the supernova expands.

improve the accuracy of the parameters κ , μ and m , and hence of our estimate of the distance to M81. Together with the fully correlated data sets they may also reveal subtle opacity effects on the apparent size of the supernova and thus give clues as to the physical mechanism responsible for the absorption of the radio emission at early times. $\square$

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1. Bartel, N. in *Supernovae as Distance Indicators* (ed. Bartel, N.) 107–122 (Lecture Notes in Physics Vol. 224 Springer, Berlin 1985).
2. Bartel, N. et al. *Nature* **318**, 25–30 (1985).
3. Bartel, N. et al. *Astrophys. J.* **323**, 505–515 (1987).
4. Wilkinson, P. N. & de Bruyn, A. G. *Mon. Not. R. astr. Soc.* **242**, 529–534 (1990).
5. Bartel, N., Rupen, M. P., Shapiro, I. I., Preston, R. A. & Rius, A. *Nature* **350**, 212–214 (1991).
6. Rupen, M. P., Bartel, N. & Foley, A. R. in *ESO Workshop and Conf. Proc.* Vol. 37 (eds. Danziger, I. J. & Kj  r, K.) 645–649 (ESO, Garching, 1991).
7. Bartel, N. in *Supernovae* (ed. Woosley, S.) 503–514 (Springer, Berlin, 1991).
8. Nomoto, K. et al. *Nature* **364**, 507–509 (1993).
9. Podsiadlowski, Ph., Hsu, J. J. L., Joss, P. C. & Ross, R. R. *Nature* **364**, 509–511 (1993).

10. Trammell, S. R., Hines, D. C. & Wheeler, J. C. *Astrophys. J.* **414**, L21–L24 (1993).
11. Wheeler, J. C. et al. *Astrophys. J.* **417**, L71–L74 (1993).
12. McCall, M. L. *Mon. Not. R. astr. Soc.* **210**, 828–837 (1984).
13. Marscher, A. P. in *Supernovae as Distance Indicators* (ed. Bartel, N.) 130–137 (Lecture Notes in Physics Vol. 224 Springer, Berlin, 1985).
14. Chevalier, R. A. *Astrophys. J.* **258**, 790–797 (1982).
15. Chevalier, R. A. *Astrophys. J.* **259**, 302–310 (1982).
16. Freedman, W. L. et al. *Astrophys. J.* (in the press).
17. Bartel, N. et al. in *VBL Technology-Progress and Future Observational Possibilities* (eds Inoue, M., Sasao, T., Manabe, S. & Kameya, O. (URSI/IAU Symp., TERRA Scientific Publishing Company, Tokyo, in the press).
18. Schmidt, B. P. et al. *Nature* **364**, 600–602 (1993).
19. Gull, S. F. *Mon. Not. R. astr. Soc.* **161**, 47–69 (1973).
20. Slysh, V. I. *Astr. Astrophys. Trans.* **1**, 171–193 (1992).
21. Altunin, V. I. *Astr. Zh.* **55**, 559–563 (1978); translated in *Soviet. Astr.* **22**, 321–324 (1978).
22. Jauncey, D. L. et al. *Nature* **334**, 412–415 (1988).
23. Staveley-Smith, L. et al. *Nature* **366**, 136–138 (1993).
24. Weiler, K. W., van Dyk, S. D., Sramek, R. A., Panagia, N. & Rupen, M. P. in *Proc. 34th Herstmonceux Conf.: Circumstellar Media in the Late Stages of Stellar Evolution* (eds Miekele, P. & Clegg, R.) (Cambridge Univ. Press, in the press).
25. Shklovskii, I. S. *Pis'ma Astr. Zh.* **7**, 479–481 (1981); translated in *Soviet Astr. Lett.* **7**, 263–264 (1981).
26. Bandiera, R., Pacini, F. & Salvati, M. *Astrophys. J.* **284**, 134–140 (1984).
27. Wyckoff, S. & Murray, C. A. *Mon. Not. R. astr. Soc.* **180**, 717–729 (1977).
28. Bietenholz, M. F., Kronberg, P. P., Hogg, D. E. & Wilson, A. S. *Astrophys. J.* **373**, L59–L62 (1991).
29. Weiler, K. W., Sramek, R. A., Panagia, N., van der Hulst, J. M. & Salvati, M. *Astrophys. J.* **301**, 790–812 (1986).
30. Sandage, A. *Astr. J.* **89**, 621–629 (1984).
31. Rogers, A. E. E. et al. *Science* **219**, 51–54 (1983).
32. Napier, P. J. et al. *Proc. Instn. elect. electron. Engrs* (in the press).

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Direct catalytic conversion of methane to acetic acid in an aqueous medium

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ALTHOUGH methane is the most abundant of alkanes, hazards of handling and distribution prevent known methane reserves^{1,2} from being fully exploited. Moreover, it is the least reactive alkane, so whereas selective conversion to more useful chemical products would be of great value, it is difficult to achieve. A useful target molecule for methane conversion is acetic acid, but existing approaches to this conversion on an industrial scale involve many steps under extreme reaction conditions^{3–5}. Previous reports of the direct catalytic conversion of methane to acetic acid have involved peroxydisulphate as the oxidant^{6,7}, but any such process that could be adapted to large-scale applications would have to use O₂ as the oxidant. Here we describe such a process, which uses rhodium trichloride as the catalyst, proceeds in an aqueous medium at a temperature of around 100 °C, and gives high yields of acetic acid. This reaction provides a potentially useful means to convert methane directly to an industrially useful organic compound and greatly expands the scope of catalytic functionalization of alkanes.

The production of acetic acid in 1992 was 3.6×10^9 lb (ref. 8). Conversion of methane to acetic acid on an industrial scale currently involves three separate steps: (1) high-temperature steam reforming of methane to a 3:1 mixture of H₂ and CO (ref. 3); (2) high-temperature conversion of a 2:1 mixture of H₂ and CO to methanol³; and (3) carbonylation of methanol to acetic acid⁴, mainly through the Monsanto process⁵.

Our results are summarized in Fig. 1 and Table 1. In its simplest form, the catalytic process involves the reaction of a

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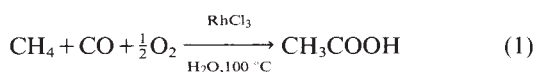
TABLE 1 Catalytic conversion of methane to acetic acid

Promoter	[H ⁺] (M)	[Cl ⁻] (M)	Temp. (°C)	Time (h)	Yields* (M)		
					CH ₃ CO ₂ H	CH ₃ OH	HCO ₂ H
None†	0.1	0.13	140	60	0.012	0.0052	0.0176
				72	0.014	0.0050	0.0252
5% Pd/C‡ (10 mg)	0.1	0.13	150	88	0.066	0.0040	0.0372
				108	0.088	0.0046	0.0592
				88	0.064	Trace	Trace
				130	0.092	Trace	0.0020
HI§ (0.025 M)	0.125	0.13	95	420	0.276	Trace	0.0100
				88	0.070	Trace	0.0168
				132	0.120	Trace	0.0366
				248	0.188	0.0020	0.0340
				352	0.275	0.0065	0.105
KI§ (0.025 M)	0.0005	0.13	95	88	0.070	Trace	0.0168
				132	0.120	Trace	0.0366
				248	0.188	0.0020	0.0340
				352	0.275	0.0065	0.105
				352	0.275	0.0065	0.105

Typical conditions: RhCl₃ (0.01 M) + HCl + promoter in 5 ml D₂O. Reactions were run in 120-ml stainless-steel bombs equipped with glass liners.

* Yields determined by ¹H NMR spectroscopy. † CH₄ (800 p.s.i.), CO (200 p.s.i.), O₂ (100 p.s.i.). ‡ 60 μmol of surface Pd atoms per g of catalyst (determined from H₂ chemisorption). § CH₄ (1,000 p.s.i.), CO (150 p.s.i.), O₂ (50 p.s.i.). || NaCl was added.

mixture of CH₄, CO and O₂ in the presence of RhCl₃ dissolved in water:



Methanol and formic acid were the only significant by-products observed in solution. No product was observed if either CO or O₂ was left out of the reaction mixture. The yield of acetic acid was substantially increased by adding either a source of I⁻ ions or 5% metallic palladium on carbon (we had earlier shown that an analogous reaction with metallic palladium-on-carbon alone as catalyst resulted in the straightforward oxidation to formic acid rather than acetic acid⁹). As Fig. 1 indicates, there was no significant decrease in catalytic activity even after 400 h of reaction time. By visual inspection, the systems remained homogeneous through the course of the reaction except, of course, when palladium on carbon was present. Only formic acid was observed when 5% metallic rhodium on carbon was substituted for RhCl₃ (compare metallic palladium-on-carbon alone as catalyst⁹). Also the addition of metallic mercury, which would be expected to amalgamate with and remove any colloidal rhodium present¹⁰, had no significant effect on the rate during the first 20 h, longer reaction times resulted in the gradual removal of all soluble rhodium species due to reduction by mercury metal. The above observations are consistent with a soluble rhodium species rather than metallic rhodium being the active catalyst for acetic acid formation. In the absence of added Cl⁻ ions, lower rates were observed. It appears that initially formed Rh(I)-carbonyl compounds may be the active species. Thus, when an aqueous solution of RhCl₃ was exposed to 1–2 atm of ¹³CO at ambient temperature, ¹³C-NMR spectroscopy revealed the formation of [Rh(CO)₂Cl]₂ (ref. 11) (δ, 182.5 ppm; J_{Rh-C} = 74 Hz). This compound is known to convert readily to [Rh(CO)₂X₂]⁻ (X = Cl, I) on the addition of an excess of the appropriate halide ion¹².

A catalyst system consisting of a mixture of RhCl₃ and I⁻ ions resembles the 'Monsanto system' for the carbonylation of methanol to acetic acid⁵; the active species there is [Rh(CO)₂I₂]⁻. The important differences are that in the Monsanto system: (1) no oxidant is used as there is no net oxidation, (2) a higher reaction temperature (~180 °C) is employed, and (3) a substantially lower pressure of CO is involved. In our case, a somewhat high pressure of CO was required to prevent catalyst degradation; a separate experiment showed that, in the absence of added CO, the Rh(I)-carbonyl species present in the reaction system were decomposed by O₂.

Mechanistic studies⁵ on the Monsanto system have shown that a Rh-CH₃ species is formed initially from methanol by way of methyl iodide (CH₃OH + HI ↔ CH₃I + H₂O), which is subsequently carbonylated to a Rh-C(O)CH₃ species and then hydrolysed to acetic acid (acetaldehyde is thought not to be an intermediate in the above process⁵). In view of the general simi-

larities between the two systems, it was important to find out whether in our system the conversion of methane to acetic acid proceeded through the intermediacy of methanol. To this end, we examined the products formed when CH₄ (1,100 p.s.i., 0.06 M solution concentration) and ¹³CH₃OH (0.06 M solution concentration) were added together as substrates. For both RhCl₃ and RhCl₃ + I⁻ systems, only CH₄ was converted to acetic acid while ¹³CH₃OH was simply oxidized to H¹³CO₂H (for example, see Fig. 2). This observation clearly indicated that the formation of a Rh-CH₃ species from methane occurred at a substantially faster rate than that from methanol (by way of methyl iodide⁵). The methanol (and its oxidized product, formic acid), formed in our system (Table 1) presumably arose through the hydrolysis of a fraction of the Rh-CH₃ species formed from methane. However, given that the back-reaction (that is, the formation of Rh-CH₃ from methanol) did not occur, the pro-

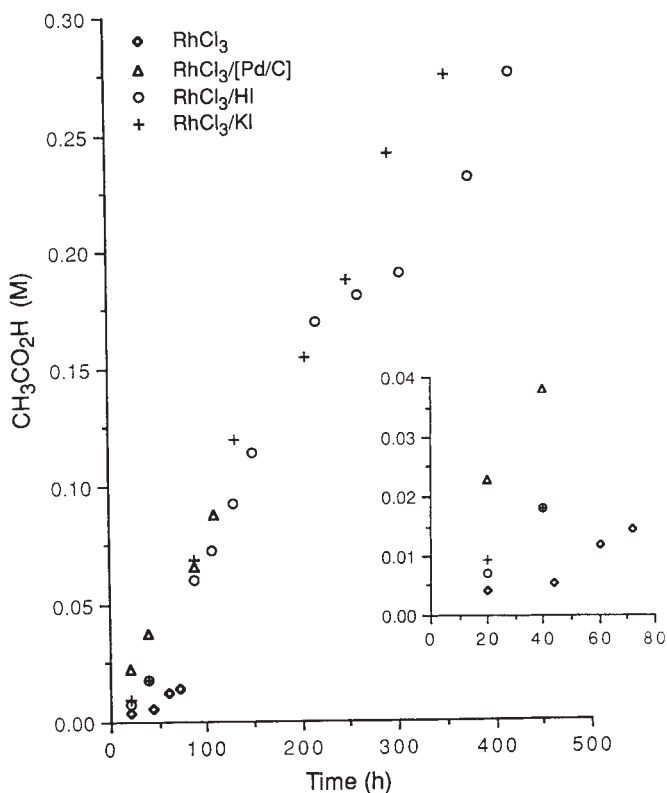
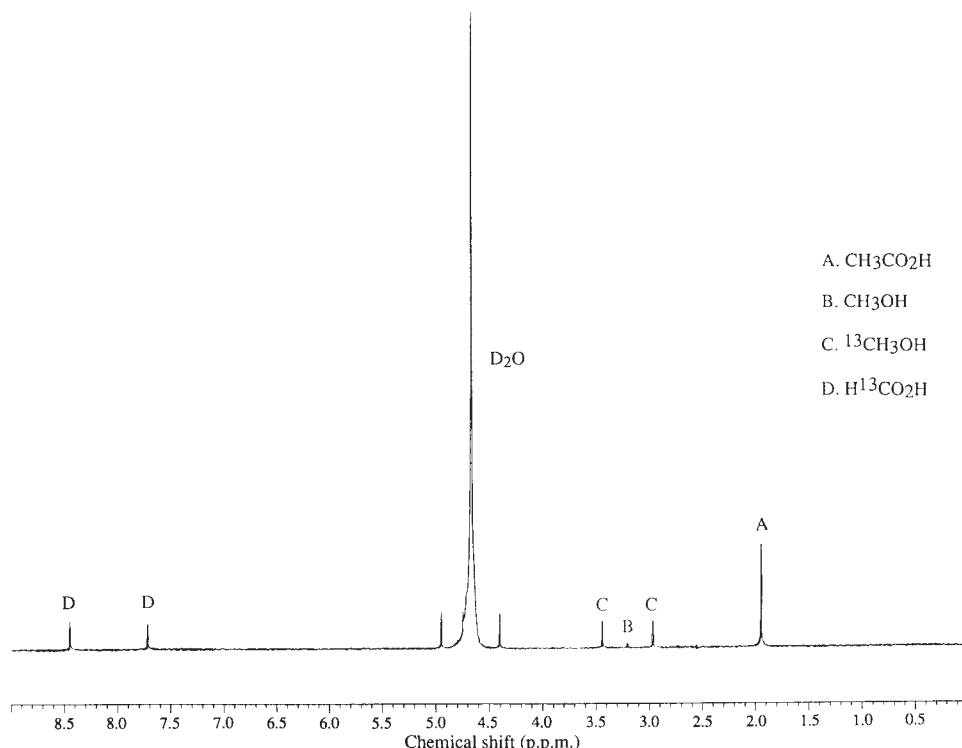


FIG. 1 Plot of the concentration of CH₃COOH formed (from CH₄) against time, obtained under the reaction conditions described in Table 1. Inset graph is an expansion showing the first 80 h of the reactions.

FIG. 2 ^1H -NMR spectrum of the product obtained after reaction between the following compounds at 100 °C for 20 h; RhCl_3 (0.01 M), HI (0.01 M), CH_4 (1,100 p.s.i., 0.06 M in water), $^{13}\text{CH}_3\text{OH}$ (0.06 M), CO (200 p.s.i.), O_2 (50 p.s.i.) and D_2O (5 ml). Chemical shifts with respect to HDO at 4.67 p.p.m.



duct distribution indicated that the hydrolysis rate was substantially slower than the rate of carbonylation to the corresponding $\text{Rh}-\text{C}(\text{O})\text{CH}_3$ species.

Although the functionalization of methane was the focus of our studies, we briefly examined ethane as a substrate. The conversion rate was significantly faster than that observed for methane, but the product specificity was lower. Acetic and propionic acids were formed in similar amounts and, in addition, a significant amount of ethanol was also found. A separate experiment indicated that the acetic acid was derived from ethanol through a subsequent oxidation step. Thus, in contrast to the $\text{Rh}-\text{CH}_3$ species, the rate of hydrolysis of the $\text{Rh}-\text{C}_2\text{H}_5$ intermediate was comparable to the carbonylation rate. These observations find parallels in the fact that the rate of hydrolysis to the corresponding alcohol was substantially faster for $[\text{Cl}_3\text{Pt}-\text{C}_2\text{H}_5]^{2-}$ than for $[\text{Cl}_3\text{Pt}-\text{CH}_3]^{2-}$ (refs 13–15; A. C. Hutson and A.S., unpublished observations).

A key question concerning the present system is the role of the I^- ion as a promoter. As methanol was not carbonylated to acetic acid under our reaction conditions, the function of the added I^- ion cannot be the same as in the Monsanto system⁵. We hope to address this and related mechanistic issues in future studies. □

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1. Masters, C. D., Root, D. H. & Attanasi, E. D. *Science* **253**, 146–152 (1991).
2. Starr, C., Searl, M. F. & Alpert, S. *Science* **256**, 981–987 (1992).
3. Wade, L. E., Gengelbach, R. B., Trumbley, J. L. & Hallbauer, W. L. in *Kirk-Othmer Encyclopedia of Chemical Technology* Vol. 15 298–415 (Wiley, New York, 1981).
4. Wagner, F. S. in *Kirk-Othmer Encyclopedia of Chemical Technology* Vol. 1 124–161 (Wiley, New York, 1978).
5. Forster, D. *Adv. Organomet. Chem.* **17**, 255–267 (1979).
6. Nishiguchi, T., Nakata, K., Takaki, K. & Fujiwara, Y. *Chem. Lett.* 1141–1142 (1992).
7. Lin, M. & Sen, A. *J. chem. Soc., chem. Commun.* 892–893 (1992).
8. *Chem. Engng. News* **71**, July 28, 41 (1993).
9. Lin, M. & Sen, A. *J. Am. chem. Soc.* **114**, 7307–7308 (1992).
10. Whitesides, G. M. et al. *Organometallics* **4**, 1819–1830 (1985).
11. Brown, C., Heaton, B. T., Longhetti, L., Povey, W. T. & Smith, D. O. *J. organomet. Chem.* **192**, 93–99 (1980).
12. Forster, D. *Inorg. Chem.* **8**, 2556–2558 (1969).
13. Sen, A., Lin, M., Kao, L.-C. & Hutson, A. C. *J. Am. chem. Soc.* **114**, 6385–6392 (1992).
14. Luinstra, G. A., Labinger, J. A. & Bercaw, J. E. *J. Am. chem. Soc.* **115**, 3004–3005 (1993).
15. Kusch, L. A., Lavrushko, V. V., Misharin, Yu. S., Moravsky, A. P. & Shilov, A. E. *Nouv. J. Chim.* **7**, 729–733 (1983).

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Avalanches and power-law behaviour in lung inflation

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WHEN lungs are emptied during exhalation, peripheral airways close up¹. For people with lung disease, they may not reopen for a significant portion of inhalation, impairing gas exchange^{2,3}. A knowledge of the mechanisms that govern reinflation of collapsed regions of lungs is therefore central to the development of ventilation strategies for combating respiratory problems. Here we report measurements of the terminal airway resistance, R_t , during the opening of isolated dog lungs. When inflated by a constant flow, R_t decreases in discrete jumps. We find that the probability distribution of the sizes of the jumps and of the time intervals between them exhibit power-law behaviour over two decades. We develop a model of the inflation process in which ‘avalanches’ of airway openings are seen—with power-law distributions of both the size of avalanches and the time intervals between them—which agree quantitatively with those seen experimentally, and are reminiscent of the power-law behaviour observed for self-organized critical systems⁴. Thus power-law distributions, arising from avalanches associated with threshold phenomena propagating down a branching tree structure, appear to govern the recruitment of terminal airspaces.

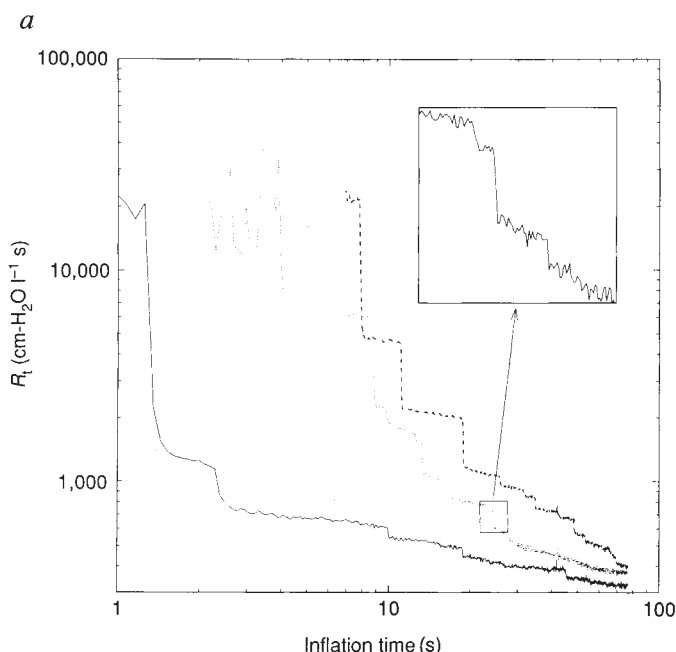
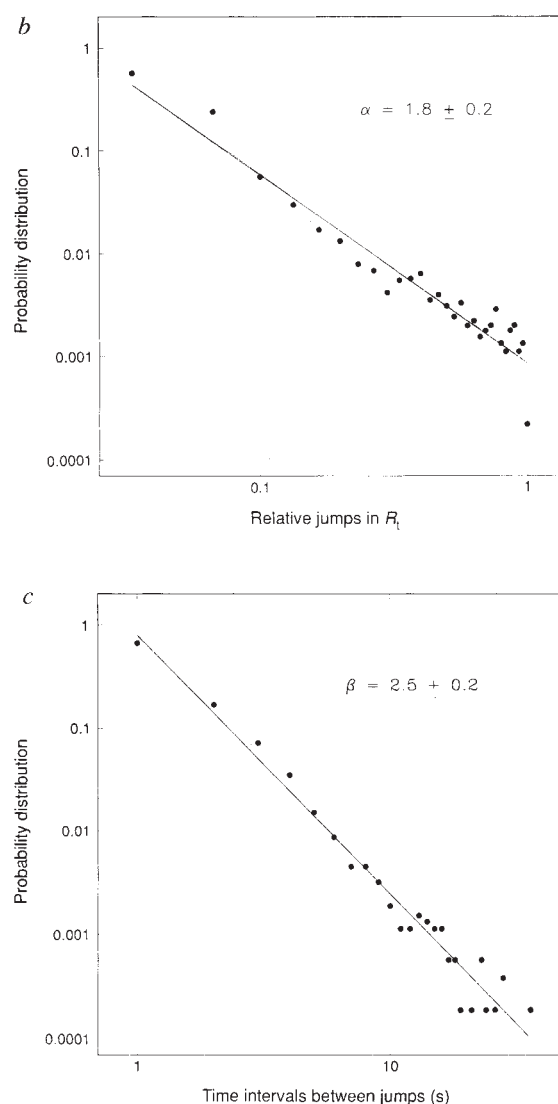


FIG. 1 *a*, The terminal airway resistance R_t as a function of inflation time for three different capsules on a single dog lung lobe. The measurements were carried out using a modification⁶ of the alveolar capsule oscillator technique⁵. Three small plastic capsules were glued to the surface of each isolated dog lung lobe studied. The pleura was punctured, so that the capsules were in communication with the alveolar space through a hole of diameter ~ 0.5 mm. The capsules were connected to a small loudspeaker-in-chamber system through polyethylene catheters. Small amplitude, sinusoidal pressure oscillations with a frequency of 10 Hz were led into the periphery of the lung through the capsules. The input and output pressures of the catheter system were measured with miniature pressure transducers in the loudspeaker chamber, and in a side tap of the capsules, respectively. The local input impedances seen from the capsules were calculated as the load impedance on the catheters. R_t was then obtained as the real part of the local impedance. Note that R_t decreases in discrete jumps as lung volume increases. The magnified portion of the curve (inset) demonstrates the existence of smaller jumps at a smaller scale. The three different line types denote R_t measured in three different capsules *b*, Probability distribution function $\Pi(x)$ of the relative jumps in R_t . We detected the smaller jumps with software that could zoom-in and magnify the desired portions of the curves as shown in *a*. This allowed us to collect from 1,000 to 2,000 discrete jumps from the 10–16 repeated

Using the alveolar capsule oscillator technique, R_t was measured during constant-air-flow inflation from residual volume to total lung capacity, TLC, in lung lobes from four dogs^{5,6}. Figure 1*a* shows that during inflation, R_t decreases in well-defined discrete jumps that are superimposed on a continuously decreasing curve. Both the sizes of the jumps and the time intervals between them show a significant variability within a single inflation. When we magnify certain portions of the curves (Fig. 1*a*, inset), we generally find further structures with smaller jumps that are statistically similar to those seen on the entire curves. The magnitudes of the jumps and the time intervals between them vary substantially among capsules. Moreover, these magnitudes and intervals are different even in one capsule when the inflation is repeated.

We quantified the statistical properties of the jumps in R_t by measuring the probability distribution functions $\Pi(x)$ and $\Pi(t)$ of the sizes of the relative jumps x in R_t and the time intervals t between the jumps. Figure 1*b* and *c* demonstrate that the statistics made over the entire data set reveal that both $\Pi(x)$ and $\Pi(t)$ display a range of nearly two decades of their arguments, over which these functions decrease linearly on a



re-inflations in each lobe from which histograms of the distribution functions were constructed. *c*, Probability distribution function $\Pi(t)$ of the time intervals between jumps in R_t . The straight lines represent the best fits to the data; their slopes give the exponents α and β .

log-log graph. We conclude that $\Pi(x)$ and $\Pi(t)$ follow power-law distributions, that is, $\Pi(x) \approx x^{-\alpha}$ and $\Pi(t) \approx t^{-\beta}$, with $\alpha = 1.8 \pm 0.2$ and $\beta = 2.5 \pm 0.2$. A power-law distribution indicates the lack of a characteristic scale; indeed, it is known that distributions describing phenomena with a characteristic size must decrease exponentially in the 'wings', as the wings represent rare events far outside this characteristic size⁷. Thus, our experimental finding of a power-law distribution for the jumps in R_t , and the time intervals between jumps, implies that the opening process is not dominated by any characteristic size scale or characteristic timescale.

Before interpreting the power-law distributions, we first note that R_t depends sensitively on the air flow resistance of the shortest pathway connecting the trachea and the terminal airway in which the measuring capsule is located—the 'primary pathway' corresponding to a given capsule. R_t is also influenced by the mechanical properties (such as airflow resistance, and the airway and alveolar wall elasticity) of the subtree comprising all those airways and the supplied alveolar regions that join the primary pathway at each generation. During inflation, the radii of the airways in the primary pathway increase, giving rise to a contin-

uous decrease in R_i . The existence of discontinuities in R_i implies that some fraction of the subtrees joining the primary path open suddenly when the intra-bronchial pressure, P_B , exceeds a threshold value. Indeed, Macklem *et al.* have demonstrated that overcoming a critical (or opening) threshold pressure is necessary to open canine bronchioli⁸, a result supported by a study in flexible-tube models⁹. Furthermore, the time required to open an airway is determined by the viscosity of the fluid film covering the airway wall; this time is well under 0.05 s for the smaller airways⁹. Thus, airways open in such a short time that our measurements detect discontinuous jumps.

To see how the discontinuous jumps in R_i lead to power-law distributions for $\Pi(x)$ and $\Pi(t)$, we assume that airways do not open independently, but rather in bursts—that is, the opening of one airway may initiate the opening of several more peripheral airways. When P_B exceeds the opening threshold pressure of an airway, all daughter airways in the subtended tree that had a smaller threshold than P_B (or were not closed) would be detected as open. The number of airways, and hence the size of the recruited alveolar volumes involved in such an opening sequence, depends on the size of the subtrees, and can vary substantially. Such a process of sequentially activating different numbers of elements triggered by overcoming a threshold is often termed an avalanche⁴. In complex dynamic systems, the existence of avalanches of very different sizes is thought to lead naturally to power-law dependences^{4,10}.

To test the above interpretation of the observed opening phenomena, we developed a branching-airway model the elements of which are circular elastic tubes of radius r and length l . The airways are assigned a generation number i ($i=0, \dots, N$; N is the order of the tree) and a column number j ($j=0, \dots, 2^i-1$). The last airways terminate in elastic alveoli represented by equal-sized spheres. An opening threshold pressure P_{ij} is also assigned to each airway (i, j). Opening of an airway occurs when P_{ij} is smaller than the pressure in its parent. Initially, P_{ij} is uniformly distributed between 0 and the value of P_B at TLC: P_B is then increased in small increments. When P_B exceeds P_{00} , the airway (0, 0) opens and its pressure is set equal to P_B . Next, the two airways at $i=1$ are examined to determine whether they can be opened with this value of P_B , that is, if $P_B > P_{10}$ or $P_B > P_{11}$. If one or both conditions are met, then the airways (1, 0) and/or (1, 1) are also opened. This opening process is then continued sequentially down the tree until no airway is found with its $P_{ij} < P_B$. This process thus defines an avalanche in the airways. When the avalanche stops, P_B is incremented and the pressures in all the open airways are updated. We iterate this process until all airways open. The development of such avalanches in a six-generation tree ($2^6=64$ alveoli) is illustrated in Fig. 2; in the actual calculations, we use 14 generations ($2^{14} \approx 16,000$ alveoli, 256 times as many).

This model is stochastic in nature: its properties are therefore studied by inflating the lung model for many different realizations of the set of opening threshold pressures P_{ij} in the airways. The R_i as a function of inflation time predicted by our model (Fig. 3a) is remarkably similar to that observed in the experiments (Fig. 1a). Moreover, the statistical properties of R_i that is, the probability distribution functions $\Pi(x)$ and $\Pi(t)$ (Fig. 3b, c) are also in good agreement with those calculated from the experimental data (Fig. 1b, c). Both functions are power laws, characterized by exponents $\alpha = 1.7 \pm 0.2$ and $\beta = 2.5 \pm 0.2$.

Our model allows us to investigate processes that cannot be measured experimentally. One such process is the recruitment of the newly opened alveolar volumes, v . We calculated the associated probability distribution function, $\Pi(v)$, and we find $\Pi(v) \approx v^{-\gamma}$, with $\gamma = 1.1 \pm 0.2$. The significance of a power-law distribution is that the probability of finding a large avalanche is much higher than it would be if the distribution were gaussian or exponential. This implies that the newly recruited alveolar volumes following an avalanche can in fact be remarkably large. For example, in the bottom right panel of Fig. 2, the last avalanche

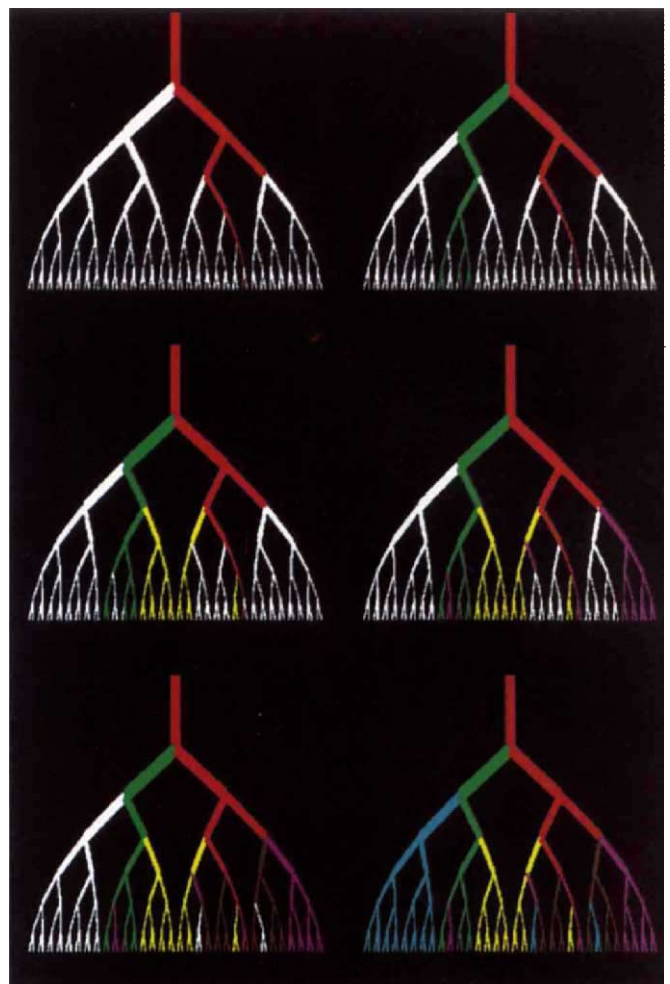


FIG. 2 The development of avalanches in a six-generation airway model. At first (top left), all airways are closed (white) whose threshold values are larger than the intra-bronchial pressure (red), which defines the first avalanche. Then the intra-bronchial pressure increases until a second threshold is exceeded, and as a result all airways further down the tree whose thresholds are smaller become inflated (green). The intra-bronchial pressure is successively increased until a third, fourth, and fifth thresholds are exceeded (yellow, magenta and brown). The last threshold to be exceeded (bottom right) results in filling the airways coloured blue. Note that the alveolar volume opened by an avalanche is proportional to the number of terminal airways that are open. Note also the wide range of avalanche sizes: the first avalanche opens only one terminal airway, whereas in the last avalanche the number of airways opened is more than a factor of ten larger than in the first avalanche, and comprises $\sim 31\%$ of the total number of airways.

che opens up over 30% of the total volume of the model, thereby significantly increasing the total alveolar surface area available for gas exchange. These findings suggest that both the magnitude and timing of pressure excursions applied at the airway entrance during artificial ventilation may be critical in triggering the avalanche process of alveolar recruitment. Furthermore, these processes may also pertain to pathological conditions of the lungs, particularly in the presence of excessive intrabronchial fluid, loss of pulmonary elasticity or increased bronchoconstrictor tone. The establishment of the relationship between the avalanche processes in the airways and various pulmonary function tests awaits further research.

Before concluding, we note that power-law distributions may

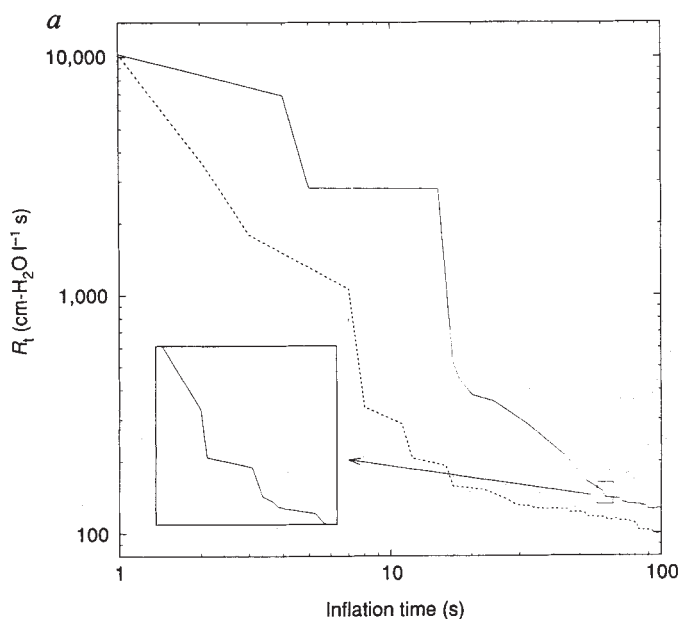
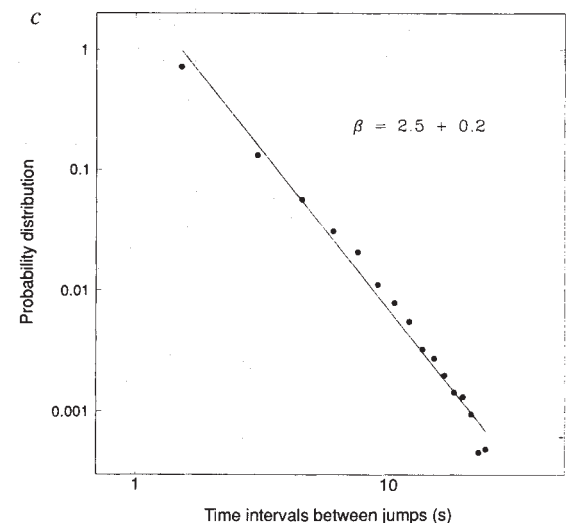
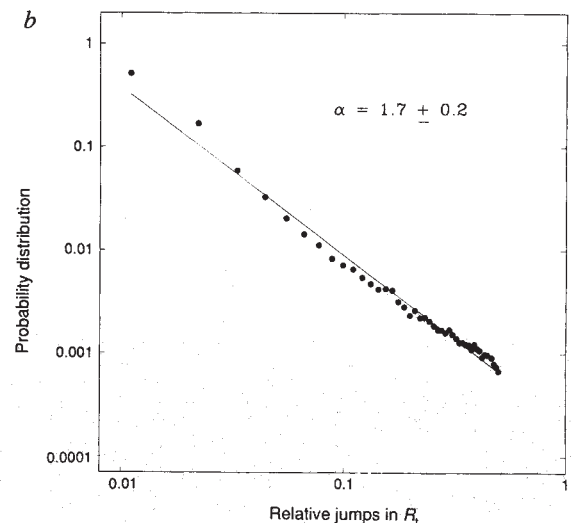


FIG. 3 Prediction of the model for the statistical properties of the terminal airway resistance, calculated for a tree comprising 14 generations. We modelled the peripheral portion of the airway tree, because it is only in these last 14 generations that airway closure was found to occur¹. The dimensions of the airways in the model were assigned by a scaling rule: the radius r_{00} and length l_{00} (see text) were chosen so that they were equal to r and l at the corresponding generation of the morphometric airway data published by Horsfield *et al.*¹⁴ The r and l were made independent of the column index j and were scaled by a factor of 0.86 and 0.9, respectively, from the r and l of the previous generation which are mean values in the Horsfield model¹⁴. Having fixed the dead space of the tree, the size of the alveoli were chosen so that the dead space was 10% of the total alveolar volume which was then uniformly distributed among the terminal airways. The airway walls and the alveolar walls were assumed to be perfectly elastic with their pressure-volume relationships similar to those described by Lambert *et al.*¹⁵ and Salazar and Knowles¹⁶, respectively. At each step of the inflation, the radii of the open branches and the alveoli were updated according to their pressure-volume relationships. Then we calculated R_t of the entire tree, seen from an alveolus, as follows. (1) The complex impedances, at 10 Hz, of all open branches—together with the impedances of the alveoli—were calculated. (2) The total terminal impedance was then obtained by marching up the primary path, starting from the terminal end and adding the next branch or alveolar impedance in the appropriate series or parallel fashion at each node. The real part of this impedance is R_t . a, Model-predicted R_t at two alveolar locations as a function of inflation time. Note the similarity between the patterns of



the discrete jumps in the model and those in Fig. 1a. The magnified portion of the curve (inset) demonstrates the existence of smaller jumps at a smaller scale. b and c, $\Pi(x)$ and $\Pi(t)$, respectively, predicted by the model (dots). The straight lines represent the best fit to the data; their slopes give the exponents α and β . We estimated these probability distribution functions from 2,000 repetitions of the inflation, requiring ~5 h calculation time for an IBM RS-6000 Model 350 work station.

be widespread in biology¹¹: for example, the static distribution of airway radii was reported to follow a power law¹². Until now, dynamic biological processes have not been studied extensively¹³,

but it is possible that the general framework of self-organized criticality⁴ will prove useful for describing a wide range of such phenomena. □

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- Hughes, J. M. B., Rosenzweig, D. Y. & Kivitz, P. B. *J. appl. Physiol.* **29**, 340–344 (1970).
- Engel, L. A., Grassino, A. & Anthonisen, N. R. *J. appl. Physiol.* **38**, 1117–1125 (1975).
- Crawford, A. B. H., Cotton, D. J., Paiva, M. & Engel, L. A. *J. appl. Physiol.* **66**, 2511–2515 (1989).
- Bak, P., Chen, K. & Creutz, M. *Nature* **342**, 780–783 (1989).
- Davey, B. L. K. & Bates, J. H. T. *Resp. Physiol.* **91**, 165–182 (1993).
- Peták, F., Hantos, Z., Adamiczka, A., Otis, D. R. & Daróczy, B. *Eur. Respir. J.* **6**, 403S (1993).
- Vicsek, T. *Fractal Growth Phenomena* 2nd edn (World Scientific, Singapore, 1992).
- Macklem, P. T., Proctor, D. F. & Hogg, J. C. *Resp. Physiol.* **8**, 191–201 (1970).
- Gaver, D. P., Samsel, R. W. & Solway, J. *J. appl. Physiol.* **69**, 74–85 (1990).
- Bak, P. & Creutz, M. in *Fractals in Science* (eds Bunde, A. & Havlin, S.) (Springer, Berlin, 1994).

- West, B. J. & Shlesinger, M. F. *Am. Sci.* **78**, 40–48 (1990).
- Shlesinger, M. F. & West, B. J. *Phys. Rev. Lett.* **67**, 2106–2109 (1991).
- West, B. J. *Fractal Physiology and Chaos in Medicine* (World Scientific, Singapore, 1990).
- Horsfield, K., Kemp, W. & Phillips, S. J. *J. appl. Physiol.* **52**, 21–26 (1982).
- Lambert, R. K., Wilson, T. A., Hyatt, R. E. & Rodart, J. R. *J. appl. Physiol.* **52**, 44–56 (1982).
- Salazar, E. & Knowles, J. H. *J. appl. Physiol.* **19**, 97–104 (1964).

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Coastal eutrophication near the Mississippi river delta

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CHANGES in delivery of river-borne nutrients such as dissolved phosphate, nitrate and silicate, owing to land-use changes and anthropogenic emissions, are known to result in eutrophication¹—enhanced phytoplankton blooms—and more severe hypoxic events^{2–4} in many enclosed bays and seas. Although similar ecological effects might be expected on continental shelves, the occurrence of such eutrophication has remained unresolved⁵. Here we present evidence of eutrophication of the continental shelf near the outflow of the Mississippi river, obtained by quantifying biologically bound silica (BSi) in diatom remnants within dated sediment cores. BSi accumulation rates are greatest in water depths of 20 to 50 m within 100 km of the river mouth, and have increased by as much as 100% this century. The increases were substantial by 1980, by which time riverine nitrogen loading had doubled relative to the beginning of the century, even though the silica loading had declined by 50% over the same period. Thus changes in river-borne nutrient loadings can modify coastal food webs and affect the amount and distribution of oxygen in bottom waters on the scale of continental shelves.

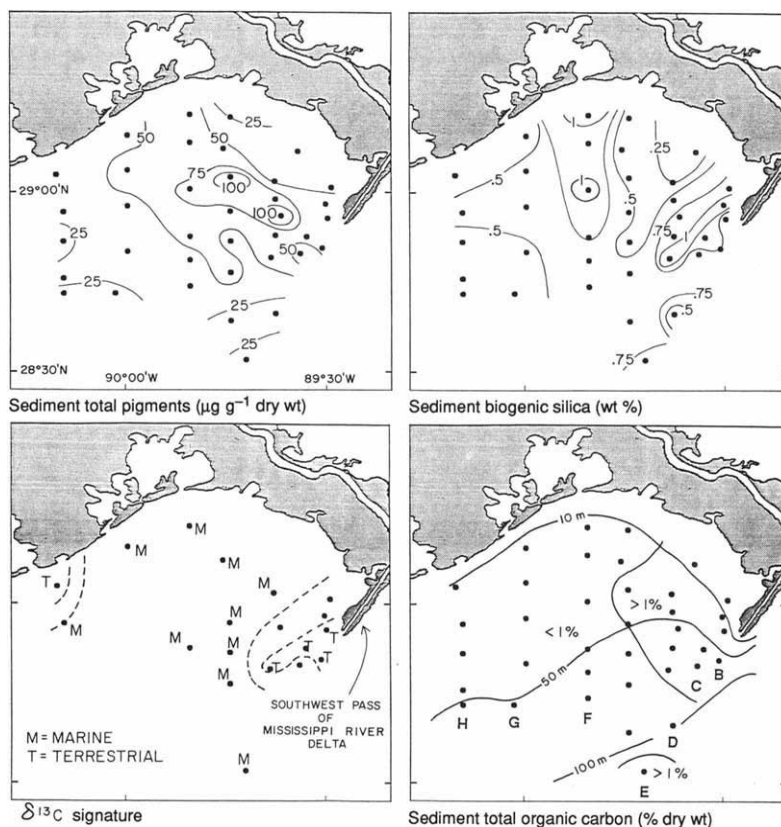
The Mississippi river, the largest river of the North American continent, drains 40% of the conterminous United States, and is third, sixth and eighth largest in the world in terms of length, sediment yield and discharge, respectively⁶. Land-use changes this century have resulted in a doubling of dissolved nitrate concentration and a halving of dissolved silicate concentration⁵.

These changes and the resulting decline in the annual average dissolved Si:N (as silicate and nitrate) atomic ratio (from 4:1 to 1:1) could have significantly affected diatom growth, intra-specific competition and production^{1,2,7}, but interpreting these results yields ambiguous answers. For example, silicate concentrations along the river-to-sea-water gradient of the Mississippi river mixing plume were analysed for samples collected after 1950. The net uptake of silicate at 30‰, towards the end of the mixing plume on the continental shelf, has remained at a similar, or perhaps at a higher level after 1979, than before 1979 (ref. 8). This result suggests that the net silicate uptake at the seawater end of the mixing plume did not decline after the 1960s when the dissolved silicate concentration fell and the dissolved nitrate concentration began to rise. In contrast, Schelske *et al.*⁹ described a relevant sequence of events in the Great Lakes, wherein increased nutrient loading (primarily phosphorus) initially stimulated diatom production rates. Diatom production subsequently declined as the silica necessary for diatom growth became limiting when the BSi was buried, rather than recycled into the water column. The new steady-state conditions resulted in lower diatom production than before eutrophication.

Many estuaries and several enclosed bays and seas (the Adriatic, the Baltic, the Black Sea and Chesapeake Bay) are known to be undergoing nutrient enrichment owing to anthropogenic influences within the watershed^{1,3,4}. But because of the substantial differences in physical characteristics and processes (such as shoreline length, flushing and wave energy) between open and enclosed coasts, it is by no means obvious that the biological effects of increased riverine nutrient loadings to open coastal waters—the case considered here—will be comparable to those seen off enclosed coasts. Two different hypotheses have been advanced⁵ to predict the possible changes in the phytoplankton community around the Mississippi outflow since the 1950s: that the productivity of coastal phytoplankton is limited by nitrogen, not silica, so that higher nitrogen loading will result in proportionally higher phytoplankton production rates; and, that the combination of lower riverine silica fluxes and a Si:N

FIG. 1 The spatial distribution in April 1989 of total phytoplankton pigments, biologically bound silica (BSi), $\delta^{13}\text{C}$ signature and total organic carbon in sediments beneath the Mississippi river delta plume.

METHODS. Surface sediments were collected by a 0.25-m² spade corer (General Oceanics). Chlorophyll *a* and phaeopigments were determined fluorometrically, before and after acidification, on the upper 5 mm of sediments after 24-h extraction in 90% acetone and combined for total¹⁹. The upper 5 cm was used for total organic carbon (Control Equipment Elemental Analyzer), BSi (ref. 20) and $\delta^{13}\text{C}$ signature (automated Deltas mass spectrometer; the Ecosystems Center, Marine Biological Laboratory, Woods Hole, Massachusetts). $\delta^{13}\text{C}$ values < -22.2‰ indicate terrestrial (T) inputs to sediments, values of -20.4 to -21.44‰ indicate marine (M) inputs, and intermediate values indicate no clear signal (unlettered) (ref. 21; B. Fry, personal communication). Letters B to H on sediment TOC diagram indicate transects.



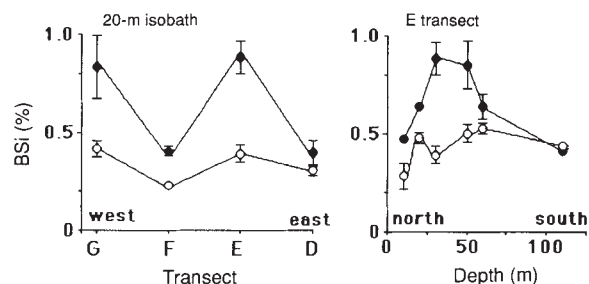


FIG. 2 The concentration of BSi in sediments along a 20-m isobath transect west of the Mississippi river delta (transects D to G) (left) and perpendicular to shore along the E transect (right). The average concentration of BSi in sediments for two different time periods is shown: between 1900 to 1960 (hollow circles), and after 1980 (solid circles). The error bar is ± 1 standard error. Values for the transition period of 1960 to 1980 are excluded. Sediments collected from 20 m water depth and ~ 20 km westward of transect H were exposed former deltaic deposits that are not presently accumulating sediments. Transect letters are the same as in Fig. 1, sediment TOC diagram.

ratio near 1:1 will result in lower phytoplankton production rates through limits on diatom production.

We investigated whether a pattern of historical eutrophication was identifiable in the continental shelf sediments influenced by the Mississippi river. We collected sediment cores from the Mississippi river delta bight (Fig. 1) during April 1989. Sample locations are from the region of locally high sedimentary carbon, sediment phytoplankton pigments from *in situ* phytoplankton production (marine origin), and BSi (1% total organic carbon (TOC), $28.1 \mu\text{g g}^{-1}$ dry weight and 0.6% BSi, respectively). This

sediment signature, directly downstream and beneath the surface riverine/estuarine dilution plume, reflects the *in situ* primary production and subsequent transport of organics from surface to bottom waters very near the river delta. Where a terrestrial signal of sediment carbon is indicated (Fig. 1), the higher concentration of BSi in surface sediments may be related to the presence of freshwater diatoms that contain ten times more silica per cell volume than marine diatoms¹⁰.

The concentration of BSi in these sediments ranged from 0.15% to 1.3%, and was highest in 25–50 m water depth (Fig. 2) in the middle of the sampling area. Sedimentation rates within 50 km of the river delta are $0.5\text{--}2 \text{ cm yr}^{-1}$ and generally $<0.3 \text{ cm yr}^{-1}$ further away from these sampling stations. Thus, the total annual deposition rates of BSi on this shelf are highest in the middle of study area. The best detail was gained using data from cores collected at intermediate depths (27–50 m), where both the BSi concentration and accumulation rates are highest (Fig. 3). Coincidental changes in the BSi concentration with time are evident, especially in the 1955 to 1965 period (a rise and fall) and a post-1975 (1980?) rise. The BSi concentration in sediments from deeper waters were generally stable through time.

The average concentration of BSi in sediments dated after 1980 is higher than for those dated between 1900 and 1960 (Figs 2–4). Sediments dated before 1900 have, on average, the same concentration of BSi as those dated between 1900 and 1960 (Fig. 4). The enhancement of BSi concentration in 15 sediment cores dated after 1980 is up to 43% higher than in sediments dated between 1900 and 1960.

The general pattern that emerges is an equilibrium accumulation of BSi from 1800 to 1900, then a slow rise, followed by a more dramatic rise in the past two decades. Coincidental variability about decadal averages exists among the sampled environments, implying shelf-wide water mass mixing and conservative accumulation of buried diatom frustules. The pattern in changes of BSi concentration parallels the documented increases in nitrogen loading in the lower Mississippi river (Fig. 3c). But nutrient data are sparse before the 1950s, and exist for only four other years (1905–06 and 1933–34) this century.

The organic carbon accumulation rate in the middle of these sample sites during the 1980s is $90 \text{ g-C m}^{-2} \text{ yr}^{-1}$ (the estimate is based on sedimentation rates and % carbon of the sediment cores), or $\sim 31\%$ of the estimated annual phytoplankton production rate of $290 \text{ g-C m}^{-2} \text{ yr}^{-1}$ (ref. 11). If the assumption is made that the BSi:C ratio at the time of deposition remained constant this century, then the increased BSi deposition after 1980 represents $\sim 40 \text{ g-C m}^{-2} \text{ yr}^{-1}$, and is thus a significant change in carbon deposition rates.

The ecological implications of the increased BSi deposition rates since 1980 include effects on continental shelf food webs and on bottom waters with severe oxygen deficiency. The effects on the diatom community of increased riverine loading of nitrogen probably compensated for the decreased loading of silica. Because the altered N:P:Si atomic ratios and loadings are known to differentially confer competitive advantages among

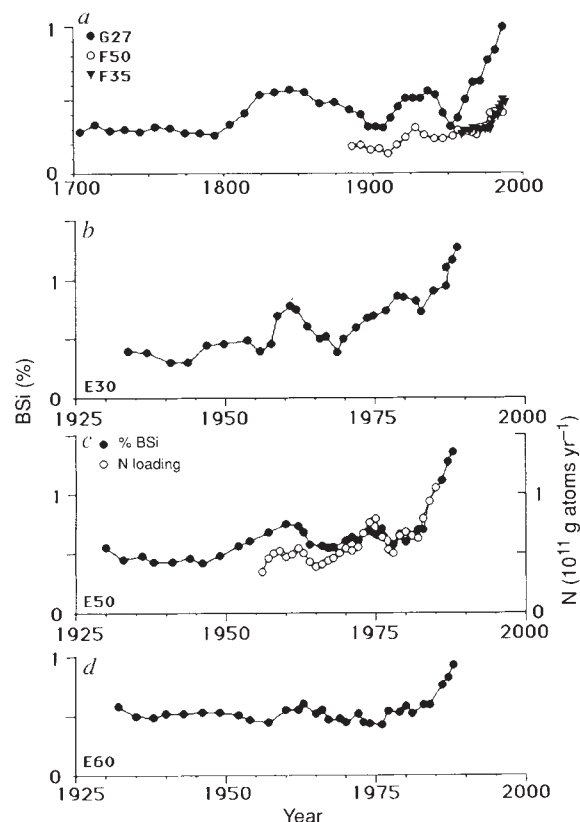


FIG. 3 The average concentration of BSi in sediments in each section of six dated sediment cores from stations at intermediate station depths (between 27 and 50 m). The letter and number code for each station are the transect and station depth, respectively. a, Data for three cores from transect F and G for the periods shown. b, c, d, Data for cores from transect E for 1925 to present, at depths of 30, 50 and 60 m, respectively. A 3-yr running average for each sampling data is shown. The data for station E50 (c) are plotted with the 3-yr running average of the nitrogen loading from the Mississippi river through the delta passes⁵. Transect letters are the same as in Fig. 1, sediment TOC diagram.

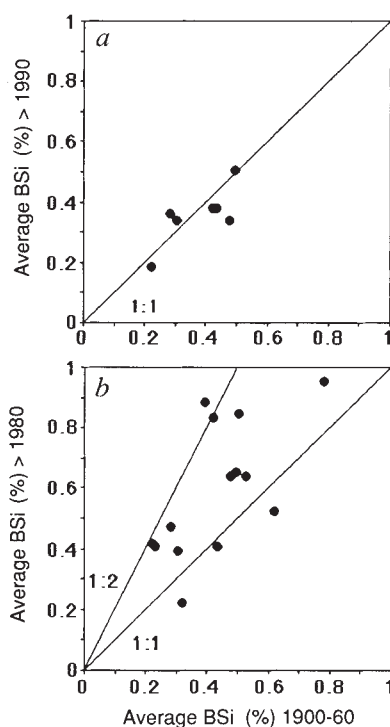


FIG. 4 The concentration of BSi in sediments dated in one interval compared to that in other intervals. *a*, Plot of the average BSi concentration of sediments dated before 1900 against that of sediments dated between 1900 and 1960 (8 stations). *b*, Plot of the average BSi concentration of sediments dated after 1980 against that of sediments dated between 1900 and 1960 (15 stations).

various diatoms and non-diatoms^{1,2,12}, these changes should affect both phytoplankton consumers and the rest of the continental-shelf food web. The implication of these results for interpreting the probability of increased severity of hypoxic (dissolved oxygen <2 mg l⁻¹) events and/or duration is particularly significant. Hypoxic water masses during the summer extend over an area of up to 9,000 km² on the inner continental shelf, and represent the largest hypoxic zone in the Western Atlantic^{13,14}. This oxygen deficiency is due both to the effects of water column stratification that restricts vertical reaeration and to the respiration of organic material derived mostly from *in situ* sources (for example, phytoplankton). These hypoxic areas have low densities of fish and invertebrate fauna¹⁴⁻¹⁸. Diatom remains are extensive in surface sediments and in bottom layer sediment traps located 30 km west of the study area (N. Qureshi and Q. Dortch, personal communication; N.N.R., unpublished data). These remains are derived directly or indirectly from *in situ* phytoplankton production. We conclude from these analyses that the organic flux of diatoms from surface to bottom waters beneath the Mississippi river plume increased this century. These changes coincided with changes in riverine nitrogen loadings and resulted in higher organic sedimentation in bottom-water layers. The depletion of bottom-water oxygen and its persistence and areal coverage on this shelf is thus indicated to have been altered this century. Because of the close coupling between riverine loading and phytoplankton production, reversal of these effects is possible to the degree that water quality changes are altered. However, the management of one nutrient (Si or N) may not be sufficient to reduce eutrophication to an acceptable amount if the compensatory qualitative adaptations of species lead to new phytoplankton communities, including those with noxious or toxic species. □

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- Smayda, T. J. in *Toxic Marine Phytoplankton* (eds Graneli, E., Sundström, B., Edler, R. & Anderson, D. M.) 29–40 (Elsevier, New York, 1990).
- Officer, C. B. & Ryther, J. H. *Mar. Ecol. Prog. Ser.* **3**, 83–91 (1980).
- Justic, D., Legovic, J. & Rottini-Sandri, L. *Estuar. Coast. Shelf Sci.* **25**, 435–445 (1987).
- Andersson, L. & Rydberg, L. *Estuar. Coast. Shelf Sci.* **26**, 559–579 (1988).
- Turner, R. E. & Rabalais, N. N. *BioScience* **41**, 140–147 (1991).
- Milliman, J. D. & Meade, R. J. *Geol.* **91**, 1–21 (1983).
- Dortch, Q. & Whitledge, T. E. *Continental Shelf Res.* **12**, 1293–1309 (1992).
- Turner, R. E. & Rabalais, N. N. *Proc. Joint Estuarine Coastal Sci. Ass. Estuarine Res. Fed.* (ed. Dyer, K. R.) (Int. Symp. Ser., Olsen & Olsen, Fredenberg, Denmark, in the press).
- Schelske, C. L., Stoermer, E. F., Conley, D. J., Robbins, J. A. & Glover, R. M. *Science* **222**, 320–322 (1983).
- Conley, D. J., Kilham, S. S. & Theriot, E. *Limnol. Oceanogr.* **34**, 205–213 (1989).
- Sklar, F. H. & Turner, R. E. *Contr. mar. Sci.* **24**, 93–106 (1981).
- Egge, J. K. & Aksnes, D. L. *Mar. Ecol. Prog. Ser.* **83**, 281–289 (1992).
- Rabalais, N. N., Turner, R. E., Wiseman, W. J. Jr & Boesch, D. F. in *Modern and Ancient Continental Shelf Anoxia* (eds Tyson, R. V. & Pearson, T. H.) 35–47 (Spec. Publ. No. 58, Geol. Soc. Lond., 1991).
- Boesch, D. F. & Rabalais, N. N. *Modern and Ancient Continental Shelf Anoxia* (eds Tyson, R. V. & Pearson, T. H.) 27–34 (Spec. Publ. No. 58, Geol. Soc. Lond., 1991).
- Harper, D. E., McKinney, L. D., Salzer, R. R. & Case, R. J. *Contr. mar. Sci.* **24**, 53–79 (1981).
- Leming, T. D. & Stuntz, W. *Nature* **310**, 136–138 (1984).
- Pavela, J. S., Ross, J. L. & Chittenden, M. E. Jr, *Northeast Gulf Sci.* **6**, 167–173 (1983).
- Renaud, M. *Fish. Bull.* **84**, 19–26 (1986).
- Parsons, T. R., Maita, Y. & Lalli, C. M. *A Manual of Chemical and Biological Methods for Seawater Analysis* (Pergamon, New York, 1984).
- DeMaster, D. J., Knapp, G. B. & Nittrouer, C. A. *Geochim. cosmochim. Acta* **45**, 1715–1732 (1981).
- Fry, B. & Sherr, E. *Contr. mar. Sci.* **27**, 13–47 (1984).
- Nittrouer, C. A., Sternberg, R. W., Carpenter, R. & Bennett, J. T. *Mar. Geol.* **31**, 297–316 (1979).

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Worldwide occurrence of silica-rich melts in sub-continental and sub-oceanic mantle minerals

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Rock samples derived from the Earth's upper mantle commonly show indirect evidence for chemical modification. Such modification, or 'metasomatism', can be recognized by the precipitation of exotic minerals such as phlogopite, amphibole or apatite¹, and by the overprinting of the bulk compositions of the mantle rocks by a chemical signature involving the enrichment of potassium and other 'incompatible' elements². Here we study the composition of the metasomatic agents more directly by examining melt and fluid inclusions trapped in mantle minerals. These inclusions are secondary, forming trails along healed fracture planes. A systematic study of the chemical compositions and entrapment temperatures and pressures of inclusions from 14 ultramafic peridotites from both continental and oceanic intraplate regions shows that volatile- and silica-rich metasomatic melts are present throughout the lithosphere. Their compositions, which differ dramatically from those of erupted, mantle-derived magmas, are more akin to continental than to oceanic crust.

The xenolith samples come from the Society Islands (Tahaa, Tahiti), Canary Islands (Lanzarote, Hierro), Kerguelen Island (Jeanne d'Arc Peninsula), Comores Island (La Grille), New Mexico (Kilbourne Hole), Arizona (San Carlos), Germany (Dreiser Eifel), Italy (Mt Ibile), France (Massif Central), Mon-

TABLE 1 Thermodynamic parameters of inclusions in the ultramafic xenoliths

Locality	Samples	T_m (°C)	T_h^1 (°C)	ρ_{CO_2} (g cm ⁻³)	P_{CO_2} (GPa)
Tahaa	7GPi	1,220	7	0.88	0.7
Tahiti	TAH1	1,270	-17	1.02	1.0
Comores	CLG1	1,230	-8	0.97	0.9
Kerguelen	LVLK133A1-1	1,250	-37.5	1.11	1.3
Lanzarote	LA93-1	1,250	-27	1.06	1.1
Hierro	HI93-3	1,270	-7	0.97	0.9
Vietnam	VNP1	1,250	-34	1.09	1.2
Dariganga	85207	1,240	-37	1.11	1.2
Vitim	314-59	1,250	-46	1.14	1.4
Massif Central	MCB1	1,220	-35	1.10	1.2
Dreiser Eifel	D5W 8906	1,250	-38.5	1.11	1.3
Mt Ibilei	IBL1	1,250	-42	1.12	1.3
Arizona	SNC 8309	1,260	-24	1.05	1.1
New Mexico	KLB 8312	1,240	-28	1.07	1.1

T_m , final melting temperature ($\pm 20^\circ\text{C}$) of the daughter minerals enclosed in melt inclusions; T_h^1 , homogenization temperature ($\pm 0.5^\circ\text{C}$) into the liquid phase for CO_2 fluid inclusions; P_{CO_2} , minimum trapping pressures for CO_2 inclusions³; ρ_{CO_2} , density of pure CO_2 in fluid inclusions.

golia (Dariganga), Russia (Vitim Highland) and Vietnam. They are massive anhydrous spinel-lherzolites and harzburgites, with 60–81% olivine, 10–21% orthopyroxene, 0.5–11% clinopyroxene and 0.5–3% spinel. Textures vary from protogranular to porphyroclastic, often with a transition between the two. The coarse-grained protogranular texture is characterized by olivine and orthopyroxene occurring as large crystals (up to 1 cm across), often with kink-banding and deformation lamellae. Irregularly shaped spinel grains occur in intergrowth with orthopyroxene and clinopyroxene. In the porphyroclastic-textured xenoliths, spinel occurs as small grains at grain triple junctions.

All the xenoliths contain secondary melt inclusions in olivine, orthopyroxene and clinopyroxene. Their distribution outlines healed fracture planes and sometimes cuts across grain boundaries, a phenomenon typical of secondary inclusions³. Their shape is irregular (round to sigmoidal) and their size is generally $< 30\ \mu\text{m}$. Most of the inclusions contain one or more gas (with or without liquid) bubbles. Some inclusions are composed only of glass, but generally they contain micrometre-size daughter minerals, crystallized from the trapped melt^{4,5}. The most common daughter minerals are K-rich amphibole (kaersutite), diop-

side, rutile, ilmenite and carbonate (magnesite), a paragenesis clearly different from the host peridotite paragenesis.

Fluid inclusions occur as secondary inclusions in olivine and pyroxene in the ultramafic xenoliths. Their shape is sub-spherical, $< 20\ \mu\text{m}$ across, and they contain CO_2 (both liquid and vapour). A co-genetic relationship between melt inclusions and fluid inclusions is revealed by: (1) the systematic association of melt inclusions and fluid inclusions along the same trails; (2) the occurrence of multiphase inclusions consisting of both CO_2 -rich fluid and melt; (3) the occurrence of melt inclusions and CO_2 inclusions joined by necks.

Heating-stage experiments were conducted on glass inclusions and melt inclusions with different degrees of crystallization, to estimate the temperature of entrapment and the initial composition of the melt. The complete homogenization of the inclusions, that is the disappearance of the shrinkage bubble, could not be obtained even at temperatures $> 1,350^\circ\text{C}$ because of the systematic presence of dense CO_2 bubbles (liquid/vapour) in the melt during the experiments. The final melting of the enclosed minerals always occurs between 1,220 and $1,270^\circ\text{C}$ (Table 1). Liquidus temperatures of the trapped melts can be considered close to these final melting temperatures^{6,7}.

Melting temperatures for fluid inclusions on the cryometric stage are close to the CO_2 triple point of -56.6°C , indicating that the trapped fluid is nearly pure CO_2 . This allows fluid density determinations using the homogenization method based on the CO_2 vapour-liquid equilibrium³ (Table 1). The maximum density for the CO_2 inclusions in oceanic xenoliths varies from $0.88\ \text{g cm}^{-3}$ for Tahaa to $1.11\ \text{g cm}^{-3}$ for Kerguelen, corresponding to homogenization temperatures into the liquid phase (T_h^1) of 7°C and -37.5°C respectively. For continental xenoliths, density varies from 1.05 (Arizona with $T_h^1 = -24^\circ\text{C}$) to $1.14\ \text{g cm}^{-3}$ (Vitim with $T_h^1 = -46^\circ\text{C}$), in agreement with previous work^{8–13}. From the density values and the P–V–T equation¹⁴, we obtain trapping pressures for CO_2 inclusions ranging from 0.7 GPa to 1.4 GPa at $1,250^\circ\text{C}$ (Table 1). These values represent a lower limit for entrapment pressure, because fluid inclusions may have been re-equilibrated. Evidently, trapping of CO_2 as inclusions, and thus of associated melts, occurred at upper-mantle depths.

The compositions of heated melt inclusions and unheated glass inclusions that contained no daughter minerals were determined using an electron microprobe (Table 2). The precision for chlorine and sulphur analyses was evaluated by replicate analyses of glass standards¹⁵. Unheated and heated inclusions have similar compositions, indicating that the heating experiment did not produce artefacts by the interaction between inclusions and host minerals. In addition, the homogeneity of the compositions for the heated inclusions indicates that the enclosed minerals are indeed 'daughter minerals', grown from the trapped melt, and disagrees with hypothetical post-entrapment evolution affecting the chemistry. The observed compositional homogeneity from rim to core indicates that glass inclusions are unzoned. Normative compositions of the melts are characterized by high-feldspar and quartz components (Fig. 1). The major element compositions are characterized by high contents of SiO_2 (54.3–66.0%), Al_2O_3 (15.6–22.4%), Na_2O (2.2–7.4%), K_2O (1.0–6.9%), and low values for TiO_2 (0.1–2.2%), FeO (0.7–5.0%) and MgO (0.6–4.1%). CaO contents vary from 1.4 to 11.0 wt%. High K_2O contents of these silica-rich melts cannot be derived from the xenolith minerals (olivine, clinopyroxene, orthopyroxene and spinel), which do not contain significant amounts of K_2O . Turning to volatile elements, chlorine concentrations in melts are always $> 1,000\ \text{p.p.m.}$ (up to $9,500\ \text{p.p.m.}$ in Comores island), whereas sulphur contents never exceed $500\ \text{p.p.m.}$ The occurrence of CO_2 in shrinkage bubbles of the melt inclusions, as indicated by cryometric experiments, and the persistence of CO_2 bubbles during the heating experiments, indicate a CO_2 oversaturation of the inclusions. Crystallization of numerous hydrous phases in melt inclusions indicates the presence of H_2O in the

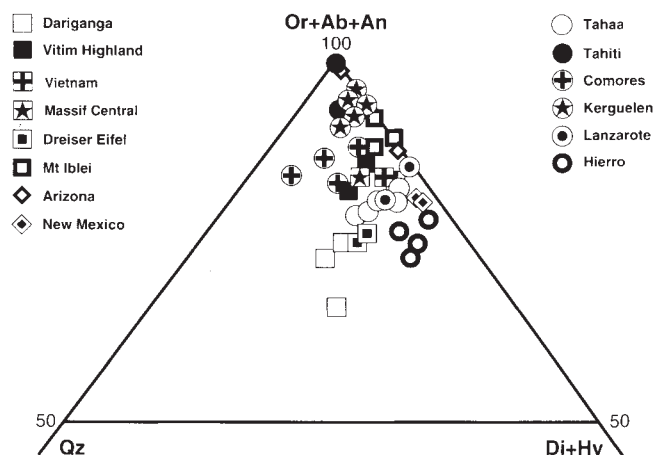


FIG. 1 Normative compositions of glass inclusions trapped in ultramafic xenoliths. Or, orthoclase; Ab, albite; An, anorthite; Qz, quartz; Di, diopside; Hy, hypersthene.

TABLE 2 Selected representative analyses of glass inclusions in oceanic and continental ultramafic xenoliths

	Society Islands				Comores		Kerguelen		Canary Islands				Vietnam (VNP1)			
	Tahaa (7GPI)		Tahiti (TAH1)		La Grille (CLG1)		Jeanne d'Arc (LVLK133A1-1)		Hierro (H193-1)		Lanzarote (LA93-1)					
SiO ₂	62.63	62.84	54.60	55.54	63.02	65.19	59.11	59.30	57.43	59.09 (0.35)	56.45	54.98	56.21	56.69		
TiO ₂	1.26	0.69	0.83	1.03	0.15	0.11	0.87	0.55	0.18	0.26 (0.02)	0.15	0.04	1.50	1.55		
Al ₂ O ₃	15.73	16.83	21.53	22.82	18.07	19.04	21.56	21.34	20.30	18.96 (0.13)	21.15	21.88	20.25	19.80		
FeO	3.50	3.47	3.04	2.25	1.04	0.97	0.82	0.82	3.29	3.21 (0.11)	2.23	2.30	2.53	2.83		
MnO	0.06	0.02	0.04	0.05	ND	ND	0.02	0.08	0.02	0.05 (0.01)	0.05	0.05	ND	ND		
MgO	2.65	3.04	3.91	2.39	1.46	0.60	1.74	1.20	2.78	3.36 (0.18)	1.36	2.06	3.43	3.52		
CaO	1.39	1.61	2.64	2.82	2.71	2.02	4.89	4.62	9.95	8.67 (0.19)	8.83	7.84	5.54	5.37		
Na ₂ O	4.02	4.17	6.26	5.47	6.46	5.38	5.79	5.56	3.84	4.29 (0.02)	4.72	6.20	4.35	4.25		
K ₂ O	6.69	6.81	4.78	5.07	3.97	4.89	4.38	4.50	0.95	1.14 (0.05)	2.12	2.32	3.44	3.20		
P ₂ O ₅	0.17	0.02	0.30	0.38	0.18	0.27	0.45	0.40	0.10	0.12 (0.02)	0.14	0.16	0.91	0.81		
Cl p.p.m.	1410	1500	1430	1810	9500	7970	1210	1150	1730	1240 (140)	3020	3360	1260	1140		
S p.p.m.	<100	<100	<100	300	350	<100	320	200	<100	<100 (50)	250	480	250	200		
SUM (%)	98.25	99.65	98.04	98.00	98.03	99.28	99.80	98.49	99.01	99.27	97.52	98.26	98.30	98.02		
HOST	ol	cpx	ol	ol	ol	ol	ol	opx	ol	ol	ol	ol	ol	ol		
	Fo ₉₀	Wo ₄₃	Fo ₈₈	Fo ₈₇	Fo ₉₀	Fo ₉₁	Fo ₉₁	Wo ₁	Fo ₉₁	Fo ₉₁	Fo ₉₁	Fo ₉₁	Fo ₉₂	Fo ₉₁		
		En ₅₁						En ₈₉								
	Mongolia Dariganga (85207)				Russia Vitim (314-59)		France Massif Central (MCB1)		Germany Dreiser Eifel (D5W 8906)		Italy Mt Iblei IBL1		Arizona San Carlos (SNC 8309)		New Mexico Kilbourne Hole (KLB 8312)	
SiO ₂	54.72	55.38	59.04	58.96	55.51	55.02	56.77	57.53	54.45	53.69 (0.25)	56.04	55.90	55.71	54.64		
TiO ₂	1.78	1.36	0.77	0.84	0.76	0.89	1.84	1.70	1.40	1.24 (0.07)	0.79	0.90	1.46	1.64		
Al ₂ O ₃	21.46	22.19	19.68	19.81	22.28	22.17	18.15	17.88	22.33	23.36 (0.25)	22.27	22.81	20.09	20.85		
FeO	4.95	4.93	2.68	2.64	2.32	2.89	3.57	3.42	1.68	1.72 (0.08)	1.21	0.88	3.06	3.40		
MnO	ND	ND	0.03	0.03	0.02	0.04	0.11	0.11	0.05	0.18 (0.09)	ND	ND	ND	ND		
MgO	2.34	2.42	2.18	2.47	1.92	1.94	2.32	2.60	0.87	0.79 (0.02)	4.02	2.59	3.04	2.15		
CaO	6.89	7.18	3.91	4.34	5.67	5.45	8.55	8.25	7.81	7.00 (0.03)	4.36	4.56	8.49	9.84		
Na ₂ O	3.38	2.18	6.21	5.67	5.56	5.60	3.50	3.79	7.20	6.86 (0.45)	4.42	5.20	4.87	4.28		
K ₂ O	1.45	1.71	2.73	2.50	3.55	3.47	2.06	2.14	3.20	3.41 (0.08)	4.22	4.59	1.47	1.48		
P ₂ O ₅	0.63	0.81	0.39	0.41	0.22	0.18	1.02	0.97	0.19	0.17 (0.02)	0.49	0.78	0.37	0.42		
Cl p.p.m.	1210	1040	1470	1140	3870	2930	2610	2430	1650	1550 (160)	1220	1240	1660	1610		
S p.p.m.	200	350	200	<100	500	450	250	<100	<100	<100 (40)	<100	<100	200	300		
SUM (%)	97.69	98.25	97.79	97.78	97.64	98.03	98.16	98.62	99.35	98.59	97.93	98.32	98.73	98.95		
HOST	ol	ol	cpx	ol	ol	ol	ol	ol	ol	ol	ol	ol	ol	ol		
	Fo ₉₁	Fo ₉₁	Wo ₄₄	Fo ₉₁	Fo ₉₁	Fo ₉₀	Fo ₉₁	Fo ₉₁	Fo ₉₀	Fo ₉₀	Fo ₉₀	Fo ₉₀	Fo ₉₀	Fo ₉₀		
			En ₅₁													

Analyses were determined by electron microprobe, using an accelerating voltage of 15 kV, a sample current of 10 nA and an 8- μ m beam. Values in parentheses correspond to the standard deviation(s) of five analyses done from rim to core. Abbreviations for host minerals: ol, olivine; cpx, clinopyroxene; opx, orthopyroxene; Fo, forsterite content of olivine; Wo, wollastonite content of clinopyroxene; En, enstatite content of clinopyroxene. For Tahaa, Kerguelen and Vitim xenoliths, melt inclusions in different host minerals have similar compositions. This indicates that there is no genetic relationship between inclusions and host minerals. (ND, not determined.)

trapped melts ($H_2O \geq 1.2$ wt% for melt inclusions trapped in Kerguelen spinel lherzolites³). Glass inclusions in different host minerals from the same xenolith have similar compositions (Table 2). This rules out any hypothetical relationship between inclusions and host minerals, and is consistent with their secondary character. Thus, on the basis of their chemical composition, their high volatile-element contents and their daughter mineral types, melt inclusions in ultramafic xenoliths can be considered as trapped metasomatic melts. No Mg-rich metasomatic melt has been found in the xenoliths.

With regard to the origin of the trapped melts, anhydrous partial melting processes can be ruled out, because of the peculiar major-element chemistry as well as the high volatile contents. Local melting processes in xenoliths attributed to decompression effects, addition of a fluid phase, heating of the xenoliths by the host magma, and breakdown of pre-existing hydrous phases have been advocated to explain the occurrence of siliceous interstitial glasses in some xenoliths¹⁶⁻¹⁸. These mechanisms cannot account for the systematic occurrence of melt inclusions in xenoliths containing no hydrous phases, or for their secondary character, their daughter mineral paragenesis, and their high concentration of alkalis. Melt inclusions are unlikely to have been generated only by melting of the anhydrous host peridotite assemblages. They must represent part of a migrating metasomatic melt phase in the lithosphere; the high volatile-element contents of the inclusions suggest that volatiles played an important role during melt generation. Experiments have shown that mantle melts may become more silicic with added H_2O and alkalis^{29,30}. Addition of chlorine would also substantially affect the chemistry. Major-element variations could reflect differences in the initial source paragenesis.

Processes responsible for chemical enrichment within segments of the upper mantle have been the subject of considerable speculation. Enrichment by reaction with migrating small-volume partial melts in the mantle, or enrichment induced by migrating deep-seated fluids are the most frequently advocated models. Our systematic study of melt inclusions in continental and oceanic spinel lherzolites and harzburgites furnishes direct evidence for the existence of small amounts of migrating metasomatic melts originating from greater depths. These metasomatic melts are characterized by high contents of alumina, silica and volatile components. Their provenance could be related to hydrous partial melting of amphibole peridotite, pyroxenite or hydrous eclogite, or to open-system processes involving migrating deep-seated fluids and peridotites. Their widespread presence in ultramafic xenoliths indicates that mantle metasomatism is a ubiquitous mechanism, related to the worldwide occurrence of acidic melts in the lithosphere. □

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1. Dawson, J. B. in *Kimberlites II. The Mantle and Crust/Mantle Relationships* (ed. Kornprobst, J.) 290-294 (Elsevier, Amsterdam, 1984).
2. Harte, B. in *Continental Basalts and Mantle Xenoliths* (eds Hawkesworth, C. J. & Norry, M. J.) 46-91 (Shiva, Nantwich, 1983).
3. Roedder, E. *Rev. Miner.* **12**, 109-148 (1984).
4. Schiano, P., Clocchiatti, R. & Joron, J. L. *Earth planet. Sci. Lett.* **111**, 69-82 (1992).
5. Schiano, P., Clocchiatti, R., Mattioli, N., Weis, D. & Shimizu, N. *EOS* **74**, 320 (1993).
6. Sobolev, A. V., Sobolev, N. V., Smith, C. B. & Dubessy, J. in *Proc. 4th Int. Kimberlite Conf.: Kimberlites and Related Rocks* (ed. Ross, J.) 221-240 (Geol. Soc. Australia, Perth, 1988).
7. Clocchiatti, R., Weisz, J., Mosbah, M. & Tanguy, J.-C. *Acta vulcan.* **2**, 161-173 (1992).
8. Roedder, E. *Am. Miner.* **50**, 1746-1782 (1965).
9. Bilal, A. & Touret, J. *Bull. Soc. fr. Miner. Cristallogr.* **99**, 134-139 (1976).
10. Murck, B. W., Burruss, R. C. & Hollister, L. S. *Am. Miner.* **63**, 40-46 (1978).
11. Solovova, I. P., Ryabchikov, I. D., Kovalenko, V. I. & Naumov, V. B. *Dokl. Akad. Nauk SSSR* **263**, 179-182 (1982).

12. De Vivo, B., Lima, A. & Scribano, V. *Mineralog. Mag.* **54**, 183–194 (1990).
13. Hansteen, T. H., Andersen, T., Neumann, E. R. & Jelsma, H. *Contr. Miner. Petrol.* **107**, 242–254 (1991).
14. Touret, J. & Bottinga, Y. *Bull. Miner.* **102**, 577–584 (1979).
15. Metrich, N. & Clocchiatti, R. *Bull. volcan.* **51**, 185–198 (1989).
16. Frey, F. A. & Green, D. H. *Geochim. cosmochim. Acta* **38**, 1023–1059 (1974).
17. Forbes, W. C. & Starnes, R. J. *Nature* **250**, 209–210 (1974).
18. Francis, D. M. *J. Petrology* **17**, 357–378 (1976).
19. McRae, N. D. *Contr. Miner. Petrol.* **68**, 275–280 (1979).
20. Maaloe, S. & Prinzlau, I. *J. Petrology* **20**, 727–741 (1979).
21. Jones, A. P., Smith, J. V. & Dawson, B. *Geology* **91**, 167–178 (1983).
22. Comin-Chiaromonte, P., Demarchi, G., Girardi, V. A. V., Princivalle, F. & Sinigoi, S. *Earth planet. Sci. Lett.* **77**, 203–217 (1986).
23. Amundsen, H. E. F. *Nature* **327**, 692–695 (1987).
24. Francis, D. J. *Petrology* **28**, 569–597 (1987).
25. Edgar, A. D., Lloyd, F. E., Forsyth, D. M. & Barnett, R. L. *Contr. Miner. Petrol.* **103**, 277–286 (1989).
26. Siena, F., Beccaluva, L., Coltorti, M., Marchesi, S. & Morra, V. *J. Petrology spec. Lherzolites Issue* 271–290 (1991).
27. Hauri, E. H., Shimizu, N., Dieu, J. J. & Hart, S. R. *Nature* **365**, 221–227 (1993).
28. Ionov, D. A., Hofmann, A. W. & Shimizu, N. *J. Petrology* (in the press).
29. Kushiro, I. *Am. J. Sci.* **275**, 411–431 (1975).
30. Ryerson, F. J. *Geochim. cosmochim. Acta* **49**, 637–649 (1985).

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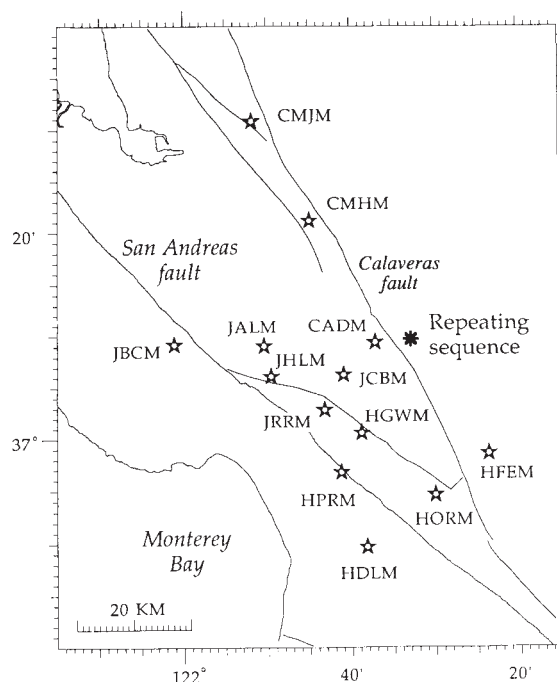


FIG. 1 Map showing the location of the 18 repeating earthquakes and the 13 Northern California Seismic Network (NCSN) stations used in this study. The depth of the earthquakes is 6 km.

Variations in rupture process with recurrence interval in a repeated small earthquake

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In theory and in laboratory experiments, friction on sliding surfaces such as rock, glass and metal increases with time since the previous episode of slip¹. This time dependence is a central pillar of the friction laws widely used to model earthquake phenomena^{2,3}. On natural faults, other properties, such as rupture velocity^{4,5}, porosity and fluid pressure^{6–11}, may also vary with the recurrence interval. Eighteen repetitions of the same small earthquake, separated by intervals ranging from a few days to several years, allow us to test these laboratory predictions *in situ*. The events with the longest time since the previous earthquake tend to have about 15% larger seismic moment than those with the shortest intervals, although this trend is weak. In addition, the rupture durations of the events with the longest recurrence intervals are more than a factor of two shorter than for the events with the shortest intervals. Both decreased duration and increased friction are consistent with progressive fault healing during the time of stationary contact.

We analyse 18 nearly identical earthquakes that occurred between July 1980 and September 1991 (Table 1). The events were about magnitude 1.5, and were on the Calaveras fault near the southern end of the magnitude-6 1984 Morgan Hill earthquake aftershock zone¹². They had identical right-lateral strike-slip mechanisms, which is usual for the Calaveras fault, and are isolated by >100 m from the nearest earthquakes of comparable or larger magnitude.

All seismograms were recorded by the short-period vertical-component stations of the Northern California Seismic Network (Fig. 1). The locations of the earthquakes centroids, or geometric centre of moment release, estimated by waveform cross-correlation¹³, all lie within 20 m. Each station recorded nearly identical records for all the events. The similarity extends from the initial P arrival throughout the S wave and the coda. Even the nodal arrivals have nearly identical waveforms. Typical cross-

correlation coefficients for recordings of multiple events at a common station are in the range of 0.95 to 1.0. The high degree of similarity allows sensitive measurement of differences between the events.

We infer that the fault is most probably creeping aseismically in the region immediately surrounding the zone, as is the case for other creeping faults in central California¹⁴. As the fault creeps in the annular zone surrounding the frictionally locked patch, it repeatedly concentrates shear stress on the same patch, which eventually fails in an earthquake.

The first of the 18 events occurred nearly 4 years before the 24 April 1984 Morgan Hill earthquake¹². The next 9 occurred in the first 7 months of the Morgan Hill earthquake aftershock sequence at steadily increasing intervals that ranged from 2.5 to 79 days. The rapid lengthening of the intervals follows the empir-

TABLE 1 Earthquake parameters

Event date	Time (UT)	Relative moment	Duration (s)	Interval (d)
26 Jul. 1980	20:04	0.92	0.037	?
26 Apr. 1984	14:28	0.95	0.027	2 or 1,370
29 Apr. 1984	04:09	0.87	0.052	3
3 May 1984	22:56	1.00	0.043	5
12 May 1984	08:37	1.07	0.033	9
27 May 1984	18:44	0.94	0.056	15
13 Jun. 1984	21:53	0.89	0.043	17
15 Jul. 1984	05:22	0.90	0.038	32
3 Sep. 1984	08:34	1.04	0.036	50
22 Nov. 1984	21:03	1.10	0.030	79
21 Oct. 1985	13:52	0.92	0.032	334
15 Jun. 1986	18:30	1.10	0.021	237
19 Jan. 1987	08:28	1.22	0.030	217
9 Feb. 1987	22:08	0.96	0.030	21
21 May 1987	04:41	0.60	0.037	99
4 Jul. 1988	13:53	1.22	0.024	410
7 Jan. 1990	17:15	1.10	0.023	552
21 Sep. 1991	10:37	1.04	0.018	648

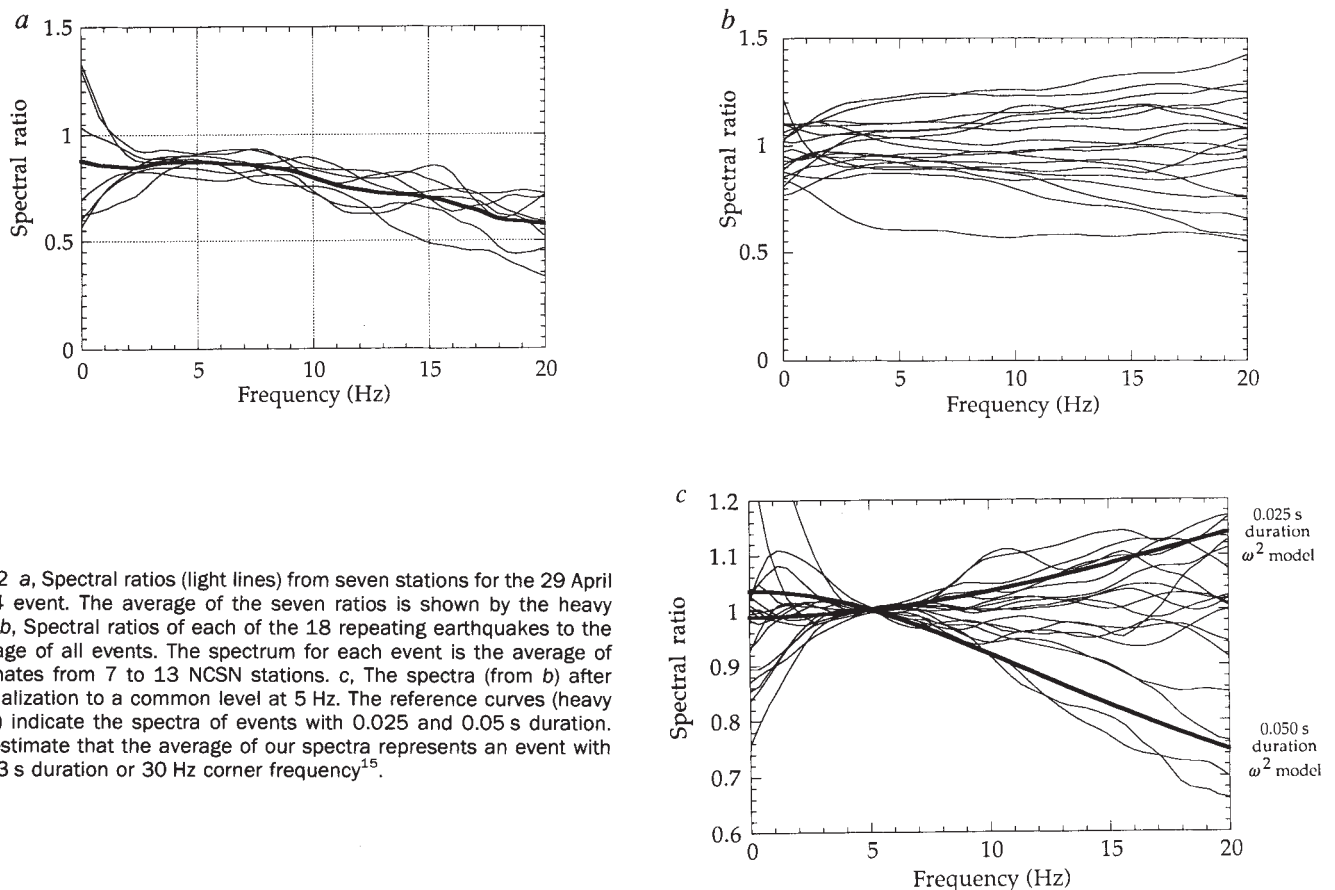


FIG. 2 *a*, Spectral ratios (light lines) from seven stations for the 29 April 1984 event. The average of the seven ratios is shown by the heavy line. *b*, Spectral ratios of each of the 18 repeating earthquakes to the average of all events. The spectrum for each event is the average of estimates from 7 to 13 NCSN stations. *c*, The spectra (from *b*) after normalization to a common level at 5 Hz. The reference curves (heavy lines) indicate the spectra of events with 0.025 and 0.05 s duration. We estimate that the average of our spectra represents an event with 0.033 s duration or 30 Hz corner frequency¹⁵.

ical decay in earthquake rate in aftershock sequences, which is inversely proportional to time elapsed from the main shock. The remaining events occurred at irregular intervals during the next 7 years.

We measure the differences between the events by calculating the ratios of spectra of individual recordings to averaged spectra for each station. This is accomplished by assembling the records of all events from a given station, aligning the first peak, and extracting a 2.56-s window. The mean and trend are removed, the ends of the window are tapered and the amplitude spectra are calculated. The station average is calculated by linearly averaging all records for each station. Finally, the spectral ratios are calculated for every station for each event. Only the most stable stations are used, and only the most noise-free traces included. About half of the available seismograms are retained. Figure 2*a* illustrates this process for an event with less than average high-frequency radiation.

Figure 2*b* shows the spectral ratio of each event to the average for all 18 events. The differences for lower frequencies, 3–7 Hz, are due to differences in seismic moment between events. The relative moments are measured from the spectral ratio amplitude at 5 Hz (Table 1). The coefficient of variation, which is the ratio of the standard of deviation to the mean, is 0.15.

We have confirmed the significance of the differences apparent in Fig. 2*b* and *c* by a similar analysis of another sequence with 13 repeating earthquakes a few kilometres away that occurred from 1984 to 1993. This second sequence of earthquakes shows much less variation in spectral slope, showing that the seismic propagation to the stations is unchanged over time, and the response of the recording system is stable. In addition, examination of the spectra of this second sequence, whose events have very short durations, and several nearby earthquakes of magnitude 0.5 to 1.0, allows us to estimate the absolute durations of the 18-event sequence, which average ~0.033 s.

The corner frequency, which is the reciprocal of the duration of faulting, is usually measured from spectra that extend well above the corner frequency. Although the corner frequencies of these earthquakes (20–50 Hz) are higher than the pass-band of our data (3–20 Hz), the high correlation between the seismograms permits accurate measurement of the subtle differences in spectral level at frequencies below the corner frequency, thus allowing us to estimate source duration. As illustrated in Fig. 2*c*, we estimate duration by comparing the observed ratio of spectral amplitudes at 5 and 15 Hz with the results for a range of ω^2 source models¹⁵ with different durations.

The durations range from 0.018 to 0.056 s, or by about a factor of three. A similar calculation with the less popular ω^3 source model¹⁵ yields a factor of about two variation in duration. Estimated from the durations and a unilateral rupture velocity of 2.5 km s⁻¹, the rupture length is 50–100 m, much larger than the centroid location of uncertainty of ± 10 m. Combining the rupture length with the magnitude of the events¹⁶ suggests ~1 cm of slip in each event. These quantities are in the range of those estimated for other earthquakes of a similar magnitude¹⁷.

Both moment and duration correlate with the time interval since the previous earthquake, as shown in Fig. 3. These results provide a glimpse of the variability during repeated rupture of the same fault patch. As moment is proportional to the product of stress drop and rupture radius cubed¹⁸, the slight increase in moment that accompanies the several order of magnitude increase in time since the last earthquake probably represents an increase of either ~15% in stress drop or ~5% in patch radius. Because the larger events have shorter durations, an increase in stress drop is more probable.

The most likely reason for an increase in stress drop with recurrence time is that the frictional strength of the fault surface increases with stationary contact time. From the available laboratory data^{2,19,20}, healing with time of stationary contact produces

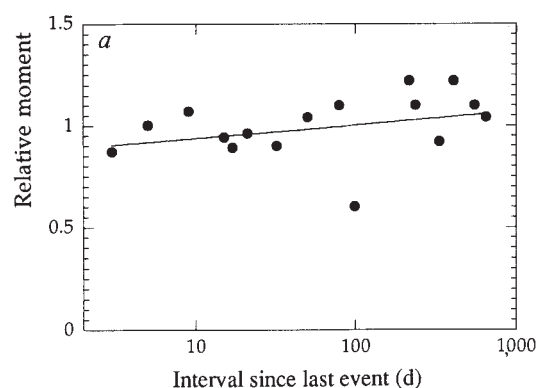
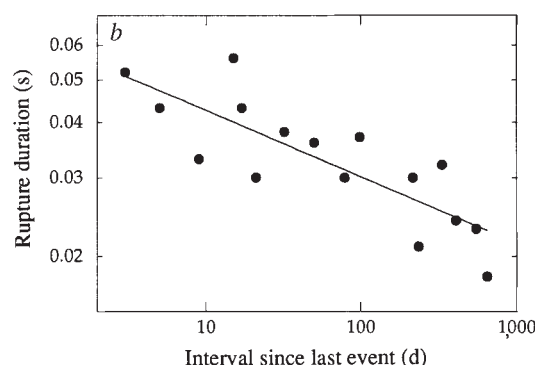


FIG. 3 a, Relative moments and b, durations of 16 events plotted against the interval since the previous repetition in the sequence. Because we cannot ascertain whether the Morgan Hill main shock broke the Calaveras fault at the location of the 16 April 1984 event, and we do not know the date of the event before that of 26 July 1980, these events are not included. The moments are derived from the spectral levels shown in Fig. 2b measured at 5 Hz. The durations of the events

about a 3–8% increase in friction for a decade change in recurrence time, in good agreement with the trend shown in Fig. 3a. So the earthquake data seem to suggest that the predictions from room-temperature, brittle experiments can be extrapolated to crustal faults. The variations in rupture duration noted below, and the observation that longer recurrence times correlate with smaller moments in the nearby 13-event sequence, however, cause this interpretation to be tentative.

It has been suggested that stress drop for earthquakes increases by a factor of 10 as the interval between subsequent events on the same fault segment increases from tens to thousands of years^{21–24}. Such long-term fault healing may be associated with slower processes, such as remineralization, which could be an effect of fluid flow in crustal fault zones^{25,26}. This rate of increase is a factor of 50 larger than our observations, and too large to be explained by the magnitude of state-dependent friction determined in short-term laboratory experiments on brittle systems.

The large decrease in rupture duration with increasing time between events is more surprising than the change in moment. It implies that either the rupture velocity or the duration of sliding at each point on the fault must be varying, if the same patch is breaking in each event, as is indicated by the virtually identical waveforms and almost constant moments. It is possible, but less likely, that the shorter-duration events could rupture a patch radius smaller by a factor of about two with an order of magnitude higher stress drop than the longer-duration events. This is not our preferred explanation because it requires a



are obtained by matching the observed spectral levels and calculated spectral levels at 15 Hz, assuming an ω^2 source model¹⁵, as illustrated in Fig. 2c. The solid lines indicate best-fit power-law curves to the data. The anomalously low moment in b is the event of 21 May 1987. We do not know why this event is anomalous. Perhaps an undetected smaller event released part of the moment before or after the anomalous event.

decrease in fault area that would have to offset the increase in stress drop almost exactly to preserve the nearly constant moments.

Laboratory experiments show that the presence of fault gouge⁴ and roughening of the fault surface⁵ reduce rupture velocity and increase the critical slip distance for seismic faulting²⁷, which plays a key role in determining the rupture nucleation dimension and thus the average rupture velocity over a small fault area. Such healing processes may account for the variation in rupture duration.

Alternatively, earthquake source models that incorporate fluid diffusion and fault compaction have been proposed^{6–11}. Such models, which do not preclude state-dependent frictional effects, have been invoked to explain the low apparent fault strength and lack of fault-zone heat flow anomalies. These models predict dramatic variation in fault-zone properties during the earthquake cycle, which have also not been previously observed *in situ*. The observed signs of changes in moment and rupture duration with changes in recurrence interval are consistent with most models of fault zone healing, although the magnitude of the duration variation is larger than expected.

Qualitatively, therefore, the observation of decreasing duration for increased time since the last event is in agreement with those laboratory experiments and models that suggest physical changes in the fault zone during the earthquake cycle. Such variations in the rupture process may play an important role in rupture initiation, the aftershock process and the earthquake cycle in general. □

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- Scholz, C. H. *The Mechanics of Earthquakes and Faulting* (Cambridge Univ. Press, 1990).
- Dieterich, J. J. *geophys. Res.* **77**, 3690–3697 (1972).
- Dieterich, J. J. *geophys. Res.* **99**, 2601–2618 (1994).
- Johnson, T. L. & Scholz, C. H. *J. geophys. Res.* **81**, 881–888 (1976).
- Okubo, P. & Dieterich, J. H. *J. geophys. Res.* **89**, 5817–5827 (1984).
- Sibson, R. H. *Nature* **243**, 66–68 (1973).
- Sibson, R. H. *Tectonophysics* **211**, 283–293 (1992).
- Byerlee, J. *Geology* **21**, 303–306 (1993).
- Blanpied, M. L., Lockner, D. A. & Byerlee, J. D. *Nature* **359**, 574–576 (1992).
- Sleep, N. H. & Blanpied, M. L. *Nature* **359**, 687–692 (1992).
- Rice, J. R. *J. geophys. Res.* (in the press).
- Bakun, W. H. *et al. Science* **225**, 288–291 (1984).
- Poupinet, G., Ellsworth, W. L. & Frechet, J. J. *geophys. Res.* **89**, 5719–5731 (1984).
- Wesson, R. L., Burford, R. O. & Ellsworth, W. L. *Stanford Univ. Publ. Geol. Sci.* **13**, 303–321 (1973).
- Aki, K. *J. geophys. Res.* **72**, 1217–1231 (1967).
- Bakun, W. H. & Lindh, A. G. *Bull. seism. Soc. Am.* **67**, 615–629 (1977).

- Abercrombie, R. & Leary, P. *Geophys. Res. Lett.* **14**, 1511–1514 (1993).
- Kanamori, H. & Anderson, D. L. *Bull. seism. Soc. Am.* **65**, 1073–1095 (1975).
- Scholz, C. H., Molnar, P. & Johnson, T. J. *geophys. Res.* **77**, 6392–6406 (1972).
- Scholz, C. H. & Engelder, T. *Int. J. Rock Mech. Mining Sci.* **13**, 149–154 (1976).
- Kanamori, H. & Allen, C. *Earthquake Source Mechanics* (American Geophysical Union, Washington DC, 1986).
- Scholz, C. H., Aviles, C. & Wesnousky, S. *Bull. seism. Soc. Am.* **76**, 65–70 (1986).
- Houston, H. *Geophys. Res. Lett.* **17**, 1413–1416 (1990).
- Kanamori, H. *et al. Bull. seism. Soc. Am.* **1993**, 330–346 (1993).
- Hickman, S. H. & Evans, B. *Fault Mechanics and Transport Properties in Rocks (the Brace Volume)* (Academic, London, 1992).
- Cox, S. F. & Paterson, M. S. *Geophys. Res. Lett.* **18**, 1401–1404 (1991).
- Marone, C. & Kilgore, B. *Nature* **362**, 618–621 (1993).

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Metabolism of polyhalogenated compounds by a genetically engineered bacterium

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THE decomposition of organic compounds by bacteria has been studied for almost a century¹, during which time selective enrichment culture has generated microorganisms capable of metabolizing thousands of organic compounds. But attempts to obtain pure cultures of bacteria that can metabolize highly halogenated compounds², a large and important class of pollutants^{3,4}, have been largely unsuccessful. Polyhalogenated compounds are most frequently metabolized by anaerobic bacteria as a result of reductive dehalogenation reactions⁵, the products of which are typically substrates for bacterial oxygenases⁶. Complete metabolism of polyhalogenated compounds therefore necessitates the sequential use of anaerobic and aerobic bacteria⁷. Here we combine seven genes encoding two multi-component oxygenases in a single strain of *Pseudomonas* which as a result metabolizes polyhalogenated compounds by means of sequential reductive and oxidative reactions to yield non-toxic products. Cytochrome P450_{cam} monooxygenase reduces polyhalogenated compounds⁸, which are bound at the camphor-binding site^{9,10}, under subatmospheric oxygen tensions⁹. We find that these reduction products are oxidizable substrates for toluene dioxygenase. Perhalogenated chlorofluorocarbons also act as substrates for the genetically engineered strain.

Separate experiments with cytochrome P450_{cam} and toluene dioxygenase identified complementary reactions that could be coupled. Cytochrome P450_{cam} reduces pentachloroethane and 1,1,1,2-tetrachloroethane to trichloroethylene and 1,1-dichloroethylene^{9,11}, respectively, which are inert to further reduction. These chlorinated products are, however, oxidized by toluene dioxygenase¹². The oxidation of trichloroethylene yields formic acid and glyoxylic acid¹³. Here we use pentachloroethane as a model substrate for characterizing genetically engineered bacteria containing both enzyme systems.

The recombinant *Pseudomonas* was constructed using the helper plasmid pRK2073 for conjugation of the *todC1C2BADE* genes, on plasmid pDTG351, into *P. putida* G786 containing the cytochrome P450_{cam} genes on the CAM plasmid. The resultant strain, *P. putida* G786 (pDTG351), constitutively expressed moderate level of toluene dioxygenase and a high level of cytochrome P450_{cam} following induction by camphor. The camphor-induced strain was incubated with pentachloroethane under anaerobic conditions. Pentachloroethane decreased immediately with a concomitant and stoichiometric accumulation of trichloroethylene (TCE) (Fig. 1b). Introduction of O₂ into the bacterial culture resulted in the depletion of trichloroethylene. The wild-type PpG786 control strain, containing only the *cam* genes, showed a very slow decline in trichloroethylene that could not be distinguished from a slow leakage of the volatile material observed in a non-biological control.

P. putida G786 (pDTG351) catalysed the oxidation of trichloroethylene more slowly than the reduction of pentachloroethane (Fig. 1b). To obtain a better balance in the rates of the two reactions, a high level of expression of toluene dioxygenase was obtained by ligating the *todC1C2BADE* genes, from pDTG601a,

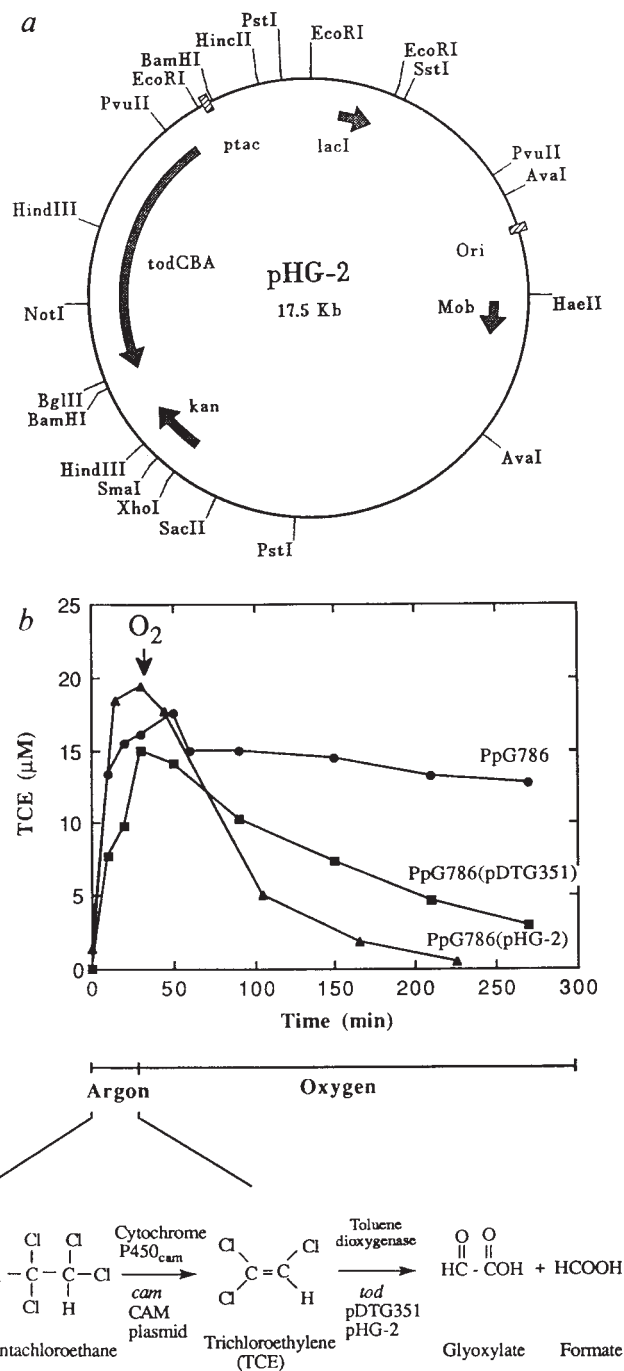
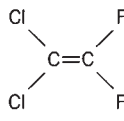
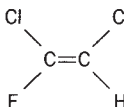


FIG. 1 Construction of plasmid pHG-2 and halocarbon metabolism by *P. putida* G786 strains with and without toluene dioxygenase genes. (a), The toluene dioxygenase genes, *todC1C2BADE*, and hybrid *trp-lac* promoter (*ptac*) were isolated as a 4.2-kb *Bam*HI fragment from plasmid pDTG601a (ref. 21) and cloned into the *Bam*HI site of plasmid pKT230 (ref. 22) to produce plasmid pHG-1. The 1.1-kb *lacI*^q gene cassette was isolated from pMMB24 (ref. 23) and cloned into the *Eco*RI site of pHG1 to produce pHG-2. Plasmid pHG-2 was transformed into *E. coli* strain DH5α and conjugated into *P. putida* G786 (ref. 24) using helper plasmid pRK2073 (ref. 14). (b), Metabolism of pentachloroethane by *P. putida* G786 (●), *P. putida* G786 (pDTG351) (■) and *P. putida* G786 (pHG-2) (▲). Cytochrome P450_{cam}-dependent reductive elimination of 20 μM pentachloroethane yielded trichloroethylene (TCE) which was oxidized under an O₂ atmosphere by *P. putida* strains containing *tod* genes. Resting-cell incubations in septum-sealed vials were done as described¹². The initial headspace gas was argon. At 30 min, O₂ was introduced with a gas-tight syringe to make up 20% of the atmosphere. Head-space chromatography is described in Table 1 legend.

TABLE 1 Oxidation of chlorofluorocarbons *in vivo* by toluene dioxygenase

Substrate		Substrate remaining (%)				
Common name	Structure	Control (sterile medium)	<i>P. putida</i> F39/D		<i>P. putida</i> G786	
			Tol [−]	Tol ⁺	−pHG2	+pHG2
Fluorochlorocarbon 1112		90 ± 3	90 ± 3	51 ± 1	100 ± 4	67 ± 14
Fluorochlorocarbon 1121		94 ± 8	86 ± 4	26 ± 12	98 ± 8	45 ± 7

P. putida F39/D was grown in the presence (Tol⁺) or the absence (Tol⁻) of toluene. Toluene induces toluene dioxygenase in *P. putida* F strains¹⁹. *P. putida* G786 strains with and without pHG2 biosynthesized and did not biosynthesize toluene dioxygenase, respectively, when grown in medium containing isopropylthiogalactoside. This was confirmed by colorimetric assay of indole oxidation to indigo²⁰. Control medium gives the background loss of substrate during sampling. Assays were done in septum-sealed vials using resting-cell suspensions as described for *P. putida* F1 (ref. 12) and *P. putida* G786 (ref. 11). Initial substrate concentration was 20 µM for *P. putida* F39/D and 50 µM for PpG786 strains. Substrate disappearance was determined by head-space gas chromatography using a Hach-Carle AGC chromatograph equipped with a flame ionization detector and an AT-1000 packed column (Altech, Deerfield, IL) operated at 180°C. Incubations were for 120 min for *P. putida* F39/D and 240 min for *P. putida* G786 (pHG2). Values are means of triplicate determinations ± s.e.

and the *lacI*^Q gene, from pMMB24, into plasmid pKT230 to construct pHG-2 (Fig. 1a). In this construct, toluene dioxygenase gene expression was controlled by the hybrid *tac* promoter. After mating into *P. putida* G786, the resultant strain metabolized pentachloroethane more rapidly and completely than *P. putida* G786 (pDTG351) (Fig. 1b).

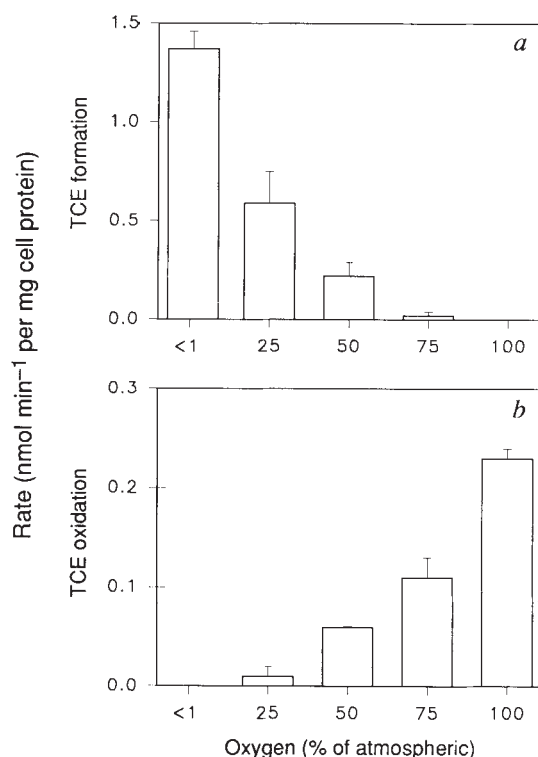


FIG. 2 Effect of oxygen on (a) trichloroethylene (TCE) formation from pentachloroethane and (b) trichloroethylene (TCE) oxidation by *P. putida* G786 (pHG-2). Incubations were done as described (ref. 12, and Fig. 1 legend). O₂ was added using a gas-tight syringe to argon-equilibrated anaerobic cell suspensions to give <1, 25, 50, 75 and 100% atmospheric O₂ concentration. Liquid and gas phases were equilibrated by vigorous mixing for 30 s. Pentachloroethane reduction and trichloroethylene oxidation was quantified¹² by determining TCE in the head-space of the vials after 5- and 15-min incubations, respectively. In a, trichloroethylene formation was undetectable at 100% O₂; in b, trichloroethylene oxidation was undetectable at <1% O₂.

As trichloroethylene was stoichiometrically formed from pentachloroethane reduction, it was used as the starting material to determine the final products of halocarbon metabolism. 1,2-[¹⁴C]trichloroethylene was incubated with *P. putida* G786 (pDTG351) and the products analysed by high-pressure liquid chromatography and CO₂ trapping as described¹³. The principal radiolabelled products in the growth medium were glyoxylic acid and 2,2,2-trichloroacetaldehyde (chloral). It had previously been shown that purified toluene dioxygenase oxidizes trichloroethylene to glyoxylic acid¹³. Formic acid, an enzyme product formed *in vitro*, was not recovered in our *in vivo* experiments. ¹⁴C-labelled CO₂ was detected at a stoichiometry of 2:1 relative to [¹⁴C]glyoxylate. This was comparable to the formate:glyoxylate stoichiometry found with toluene dioxygenase *in vitro*¹³. Controls with *P. putida* G786 and heat-killed *P. putida* G786 (pDTG351) did not yield labelled CO₂. These data indicate that the constructed strain partially oxidized pentachloroethane to CO₂ via trichloroethylene and formic acid. The strains described here did not grow on pentachloroethane or trichloroethylene as sole carbon and energy sources, but the expression of halocarbon-metabolizing genes in suitable host bacteria might provide for cell carbon and ATP synthesis. Chloral formation occurred only with camphor-induced *P. putida* G786 (pDTG351) and was dependent on cytochrome P450_{cam} monooxygenase activity. As the rate of trichloroethylene oxidation by cytochrome P450_{cam} is less than 10% of that catalysed by toluene dioxygenase, it had previously gone undetected¹⁵.

We also investigated the effect of oxygen concentration on halocarbon metabolism. Cell suspensions of *P. putida* G786 (pHG-2) were poised at <1, 25, 50, 75 and 100% of the oxygen concentration found in air, and the rates of pentachloroethane

TABLE 2 Stoichiometry of 1,1-dichloro-2,2-difluoroethylene disappearance and product formation

Strain	Substrate disappearance	Oxalic acid formation	Fluoride formation
<i>P. putida</i> G786 (pHG-2)	14.3 ± 6.0	14.5 ± 0.4	30.0 ± 1.0

Values are in micromolar and are means from triplicate determinations ± s.e. *P. putida* G786 (pHG-2) was incubated aerobically in septum-sealed vials with 200 µM 1,1-dichloro-2,2-difluoroethylene for 22 h at 23 °C. Substrate disappearance was measured as described in the legend to Table 1. Products were assayed in the culture supernatant. Oxalic acid was quantitatively determined using an organic acid column fitted to a high-pressure liquid chromatograph with absorbance detection at 210 nm. Fluoride formation was determined with a fluoride-specific electrode (Orion, Inc.) calibrated with standard NaF solutions.

reduction and trichloroethylene oxidation were measured. The reduction and oxidation reactions were inversely sensitive to O₂ concentration (Fig. 2). Oxygen concentrations intermediate between atmospheric and anaerobic supported both reactions. Pentachloroethane metabolism to trichloroethylene and its subsequent disappearance was observed at 50% atmospheric oxygen. At atmospheric oxygen concentrations, trichloroethylene was a transient intermediate when anaerobic microenvironments were provided for pentachloroethane reduction as a result of incomplete mixing. Our results have implications for metabolic reactions in soil or bioreactors, where oxygen concentrations are typically subatmospheric.

Perhalogenated compounds containing chlorine and fluorine atoms are generally resistant to attack by microbial oxygenases, but 1,1,1-trichlorotrifluoroethane is reduced by cytochrome P450_{cam} to yield 1,1-dichlorodifluoroethylene⁹. The strains described here, which express toluene dioxygenase, oxidized 1,1-dichloro-2,2-difluoroethylene and 1,2-dichlorodifluoroethylene (Table 1). Disappearance of 1,1-dichloro-2,2-difluoroethylene was accompanied by the formation of one equivalent of oxalic acid and two equivalents of fluoride (Table 2). Oxalic acid probably arises from substrate dioxygenation and subsequent *gem*² elimination and hydrolysis reactions. There were no products in incubations with *P. putida* G786, indicating that only toluene dioxygenase is responsible for substrate oxidation.

We have described the recruitment of enzymes and genes for catalysing dehalogenation reactions, complementing efforts to redesign the active site of cytochrome P450_{cam} so that it can accommodate new substrates, including halocarbons^{16,17}. Attempts to isolate a soil bacterium having the collection of genes found in *P. putida* G786 (pHG-2) were unsuccessful (E. Moe, M. S. P. Logan and L. P. W., unpublished results). This might reflect the comparatively recent entry of most polyhalogenated compounds into the natural environment. Evolutionary forces probably modify pre-existing bacterial genes to make new enzymes capable of metabolizing halocarbons. For example, 4-chlorobenzoate-coenzyme A dehalogenase, a hydrolytic dehalogenase, shares sequence similarity with the enoyl-coenzyme A hydratases that function in the β -oxidation of fatty acids¹⁸. Directed laboratory evolution, drawing on a knowledge of enzyme mechanisms and gene structure, can now be used to construct organisms for the breakdown of toxic and environmentally persistent organohalides. □

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- Stanier, R. Y., Adelberg, E. A. & Ingraham, J. L. *The Microbial World* 4th edn (Prentice-Hall, Englewood Cliffs, NJ, 1976).
- Wackett, L. P., Logan, M. S. P., Blocki, F. A. & Bao-li, C. *Biodegradation* **3**, 19–36 (1992).
- Anders, M. W. & Pohl, L. R. in *Bioactivation of Foreign Compounds* (ed. Anders, M. W.) 284–316 (Academic, New York, 1985).
- Reineke, W. in *Microbial Degradation of Organic Compounds* (ed. Gibson, D. T.) 319–360 (Dekker, New York, 1984).
- Qunson, J. F., Tiedje, J. M. & Boyd, S. A. *Science* **242**, 752–754 (1988).
- Fox, B. G., Borneman, J. G., Wackett, L. P. & Lipscomb, J. D. *Biochemistry* **29**, 6419–6427 (1990).
- Abramowicz, D. A. *Crit. Rev. Biotech.* **10**, 241–251 (1990).
- Castro, C. E., Wade, R. S. & Belser, N. O. *Biochemistry* **24**, 204–210 (1985).
- Li, S. & Wackett, L. P. *Biochemistry* **32**, 9355–9361 (1993).
- Poulos, T. L. & Raag, R. *FASEB J.* **6**, 674–679 (1992).
- Logan, M. S. P., Newman, L. M., Schanke, C. A. & Wackett, L. P. *Biodegradation* **4**, 39–50 (1993).
- Wackett, L. P. & Gibson, D. T. *Appl. Environ. Microbiol.* **54**, 1703–1708 (1988).
- Li, S. & Wackett, L. P. *Biochem. biophys. Res. Commun.* **185**, 443–451 (1992).
- Leong, S. A., Ditta, G. S. & Helinski, D. R. *J. Biol. Chem.* **257**, 8724–8730 (1982).
- Wackett, L. P., Brusseau, G. A., Householder, S. R. & Hanson, R. S. *Appl. Environ. Microbiol.* **55**, 2960–2964 (1989).
- Paulsen, M. D., Filipovic, D., Sligar, S. G. & Ornstein, R. L. *Prot. Sci.* **2**, 357–365 (1993).
- Ornstein, R. L. *Proceedings of Hazmacon* (ed. Bursztynsky, T.) 470–480 (J.T. Litho, Oakland, CA, 1991).
- Babbitt, P. C. et al. *Biochemistry* **31**, 5594–5604 (1992).
- Gibson, D. T., Zylstra, G. J. & Chauhan, S. in *Pseudomonas: Biotransformations, Pathogenesis and Emerging Biotechnology* (eds Silver, S., Chakrabarty, A. M., Iglewski, P. & Kaplan, S.) 121–132 (ASM, Washington DC, 1990).
- Ensley, B. D. et al. *Science* **222**, 167–169 (1983).
- Zylstra, G. J. & Gibson, D. T. *Genet. Eng.* **13**, 183–203 (1991).
- Bagdasarian, M. M. et al. *Gene* **16**, 237–247 (1981).
- Bagdasarian, M. M., Amann, E., Lurz, R., Rückert, B. & Bagdasarian, M. *Gene* **26**, 273–282 (1984).

- Bradshaw, W. H., Conrad, H. E., Corey, E. J., Gunsalus, I. C. & Lednicher, D. J. *Am. chem. Soc.* **81**, 5507 (1959).

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Sympatric speciation suggested by monophyly of crater lake cichlids

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THE existence of sympatric speciation—that populations diverge into species in the absence of physical or ecological barriers—is controversial^{1–6}. The East African Great Lakes harbour hundreds of cichlid species representing only a few monophyletic lineages^{7,8}, although palaeolimnological evidence^{9–11} and local restrictions on species distribution¹² suggest that speciation in these lakes could have been allopatric^{13,14}. The case for sympatry in restricted areas of Lakes Malawi and Tanganyika is stronger^{15–17} but not unassailable. A better case might be made for cichlid species flocks in small, ecologically monotonous crater lakes. Here we present a mitochondrial DNA analysis of cichlid species flocks endemic to two such lakes in Cameroon. The results suggest that the flocks in each lake are monophyletic: the implication being that each lake was colonized once only, the size and shape of each lake being such that subsequent diversification would have been sympatric.

The two volcanic crater lakes, Barombi Mbo and Bermin in Cameroon (Fig. 1a), are extremely small (4.15 and 0.6 km²) but harbour 11 and 9 endemic cichlid species, respectively. These species, which all belong to the tilapiines or tilapia-like cichlids, have been described on the basis of their morphological characters, including different breeding colorations^{18,19}. Furthermore, the species status of most of these is supported by field observations documenting assortative mating, and by the failure to find intermediate morphotypes (hybrids) despite extensive sampling of the populations. Finally, the trophic and reproductive ecology are different for each of the species judged from field observations, stomach content analyses and specialized morphological features related to feeding (refs 18–20, and U.K.S., unpublished results).

Mitochondrial DNA sequences were used to differentiate between a polyphyletic and a monophyletic origin of the species flocks. Samples were collected from all 20 cichlid species inhabiting the lakes. To achieve an adequate representation of possible ancestral species, samples were also collected from all tilapiine species existing in the neighbouring river systems and lakes. Two species considered to be particularly likely to be related to species in the lake, *Sarotherodon galilaeus* and *Tilapia (Coptodon) guineensis*, were also collected from different riverine populations. Finally, *T. busumana*, which on morphological grounds represents an early divergence among tilapiine cichlids²¹, was included in the analysis to provide an additional outgroup taxon.

A 340-base-pair fragment of the mitochondrial cytochrome *b* gene was sequenced from each species. The distance matrix (Table 1) shows that the species of Barombi Mbo are closely related to one another, differing by 0–8 transitions and 0–4 transversions. These distances are within the range of differences found for the four individuals of *S. galilaeus* sampled from populations of the adjacent rivers. All other species exhibit more sequence differences when compared with the species of Barombi

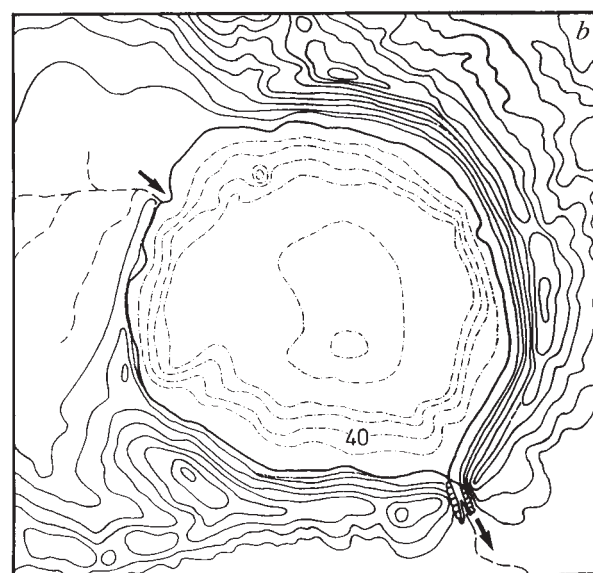
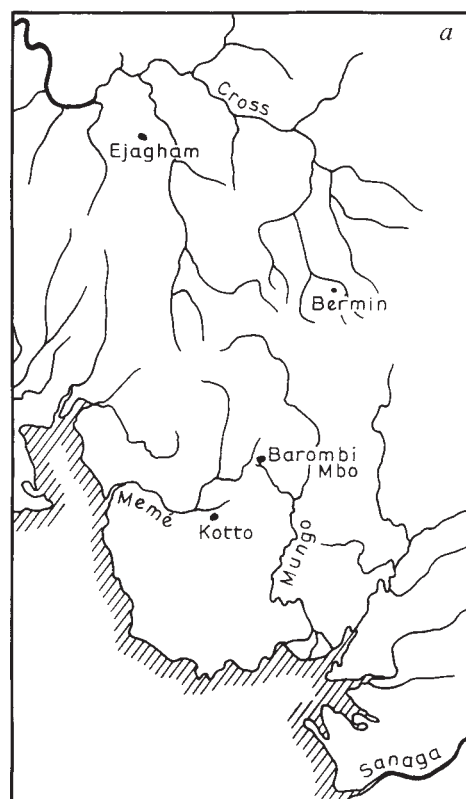
TABLE 1 Pairwise comparisons of transitions/transversions in cichlid mtDNAs

mtDNAs	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1 <i>St. mariae</i>	—	0	2	1	2	1	1	1	0	0	0	0	0	4	10	10	10	11	10	9	9	3	10	10
2 <i>St. pindu</i>	3	—	2	1	2	1	1	1	0	0	0	0	0	4	10	10	10	11	10	9	9	3	10	10
3 <i>St. mongo</i>	3	2	—	3	4	3	3	3	2	2	2	2	2	6	12	12	12	13	12	11	11	5	10	10
4 <i>S. linnellii/caro</i>	4	5	3	—	1	0	0	2	1	1	1	1	1	5	11	11	11	12	11	10	10	4	11	11
5 <i>M. myaka</i>	3	4	2	1	—	1	1	3	2	2	2	2	2	6	12	12	12	13	12	11	11	5	12	12
6 <i>S. steinbachi</i>	5	6	4	3	2	—	0	2	1	1	1	1	1	5	11	11	11	12	11	10	10	4	11	11
7 <i>S. lohbergeri</i>	3	4	2	1	0	2	—	2	1	1	1	1	1	5	11	11	11	10	11	10	10	4	11	11
8 <i>P. maclareni</i>	7	8	6	7	6	8	6	—	1	1	1	1	1	5	9	9	10	10	9	8	8	4	9	11
9 <i>K. dikume</i>	7	8	6	7	6	8	6	8	—	0	0	0	0	4	10	10	10	11	10	9	9	3	10	10
10 <i>K. eisenbrauti</i>	6	7	5	6	5	7	5	7	1	—	0	0	0	4	10	10	10	11	10	9	9	3	10	10
11 <i>S. gal. 'Meme/Cross'</i>	5	4	2	5	4	6	4	8	6	5	—	0	0	4	10	10	10	11	10	9	9	3	10	10
12 <i>S. gal. 'Ejagham'</i>	4	5	3	4	3	5	3	7	5	4	1	—	0	4	10	10	10	11	10	9	9	3	10	10
13 <i>S. gal. sanagaensi</i>	12	13	11	12	11	13	11	13	13	12	9	8	—	4	10	10	10	11	10	9	9	3	10	10
14 <i>S. m. melanother</i>	23	24	22	21	22	24	22	24	24	25	22	21	26	—	10	10	10	11	10	9	9	5	10	10
15 <i>T. Bermin A</i>	36	38	36	36	37	39	37	40	38	37	38	36	40	35	—	0	0	1	0	3	1	9	14	12
16 <i>T. Bermin B</i>	36	38	36	36	37	39	37	40	38	37	38	36	40	35	2	—	0	1	0	3	1	9	14	12
17 <i>T. guineensis 'Cross'</i>	35	35	33	35	36	38	36	39	37	36	35	35	39	34	5	3	—	1	0	3	1	9	14	12
18 <i>T. kottae</i>	34	36	34	34	35	37	35	38	38	37	36	34	38	33	8	6	5	—	1	4	2	10	15	13
19 <i>T. 'Ejagham'</i>	34	34	32	34	35	37	35	38	38	37	34	34	38	33	6	4	1	3	—	3	1	9	14	12
20 <i>T. zillii</i>	35	38	36	36	37	39	37	40	40	39	38	36	42	39	15	13	12	11	11	—	2	8	13	11
21 <i>T. g. 'Ivory Coast'</i>	33	35	33	33	34	36	34	37	35	36	35	33	35	35	23	21	20	19	19	22	—	8	13	11
22 <i>O. nil. vulcani</i>	27	28	28	27	28	30	28	26	28	27	28	26	28	32	34	34	33	32	32	31	31	—	11	11
23 <i>T. mariae</i>	32	33	32	33	32	34	32	34	36	35	35	33	33	32	41	41	40	37	39	40	38	33	—	12
24 <i>T. busumana</i>	41	42	42	40	41	43	41	45	40	41	42	41	46	41	42	44	43	42	44	44	38	43	41	—

The numbers of transversion differences are given above the diagonal and the numbers of transition differences below the diagonal for a 340-bp fragment of the mitochondrial cytochrome *b* gene. Species 1–10 from Barombi Mbo; 15 and 16 from Bermin.

Mbo (21–45 transitions, 3–13 transversions). In Bermin, all representatives of the nine species sampled show one of two cytochrome *b* sequences. These sequences differ from each other by two transitions and no transversions. When compared with populations of *Tilapia* (*Coptodon*), the Bermin species differ by

3–23 transitions and 0–3 transversions. For all other species, the Bermin sequences exhibit more differences (34–44 transitions, 9–14 transversions). Phylogenetic analysis of all species confirms that the Barombi Mbo species are closely related to one another and to the *S. galilaeus* taxa (Fig. 2a), and that the Bermin species



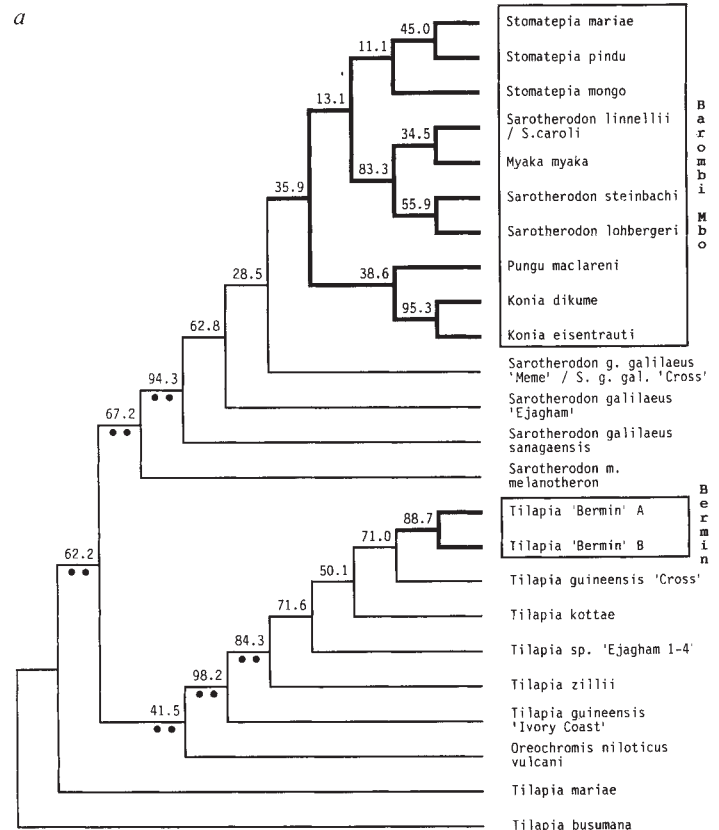
Lake Barombi Mbo

FIG. 1 Maps of a, southwestern Cameroon and b, Lake Barombi Mbo. The craterlakes from which cichlid samples were analysed are shown as well as river systems: Lake Barombi Mbo nowadays drains into the Mungo, but is believed to have drained into Memé River in the past

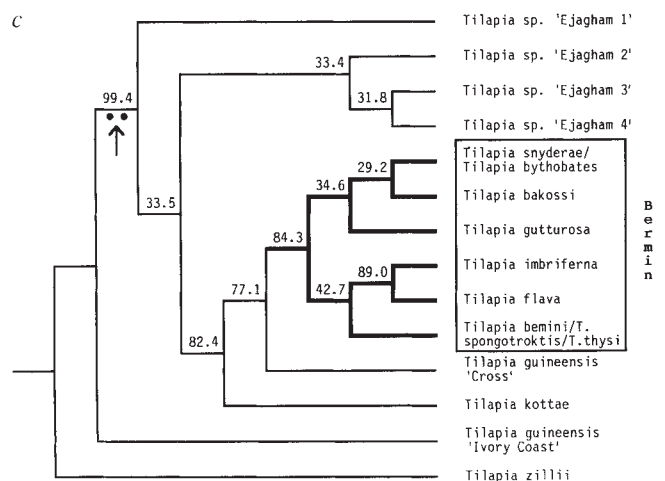
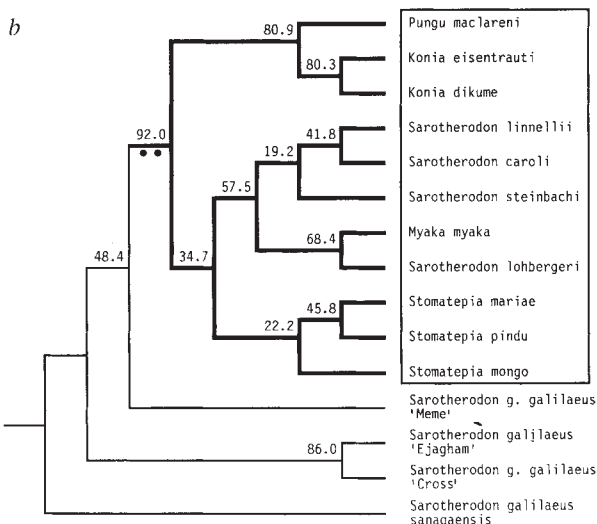
when no connection to the Mungo existed¹⁸. Lake Bermin lacks an inflow and is connected through an outflow with the Cross River System, as is Lake Ejagham. Below, a topographical map of Lake Barombi Mbo shows the homogeneous shoreline below and above water, as well as the crater rims. The small inflow to Lake Barombi Mbo lacks connection to other river systems. The areas of Lake Barombi Mbo and Bermin are 4.15 km² and 0.6 km², respectively, and their depths are 110 m and 14.5 m, respectively³¹. But, in Lake Barombi Mbo there are fish only to ~40 m depth, where the oxygenated layer ends. Inflows and outflows are marked by arrows. Maps were drawn from the 1:50,000 topographical map of Cameroon and the depth from ref. 32 for Lake Barombi Mbo.

FIG. 2 Phylogenetic trees relating the tilapiine cichlids of Barombi Mbo and Bermin with all tilapiine cichlids of adjacent rivers and lakes and tilapiine outgroups from neighbouring regions^{33,34}. *Sarotherodon galilaeus galilaeus* is represented by individuals from three populations ('Meme', 'Cross' and 'Ejagham'; despite several attempts, no *S. g. galilaeus* could be found in Mungo, where it is apparently not present). *Sarotherodon galilaeus sanagaensis* is the geographically closest subspecies different from *S. g. galilaeus*. *S. melanotheron* is the second *Sarotherodon* species in the region. The subgenus *Coptodon* is represented by *T. guineensis* from the Cross River and, as an outgroup, by a sample from Bia-Dam Lake in Ivory Coast; *T. kottae* from Lake Kotto in the Memé-River drainage, and all four tentative species from Lake Ejagham in the Cross River drainage (undescribed, except *T. deckerti*, which is *T. spec. 'Ejagham 4'*). *T. mariae* represents the single species of the subgenus *Pelmatolapia* in the area, *Oreochromis niloticus* is included because it inhabits the Niger river, which might have once been connected with the Cross River. *Tilapia busumana* from Lake Bosumtwi, Ghana, represents a basal tilapiine lineage for outgroup comparison. a, A tree relating all species based on 340 bp of cytochrome *b* sequence. Lineages shared among species in Barombi Mbo and Bermin are shown in bold. In Bermin, all nine species carried one of two cytochrome *b* sequences and in Ejagham all four tentative species carried one sequence. Further lineages found in more than one species are indicated by the localities the respective populations were sampled. b, Tree for Barombi Mbo and relevant outgroups. c, Tree for Bermin with other members of the *Coptodon* subgenus. b and c are based on the cytochrome *b* sequences as well as ~350 bp of control region sequences. Dots denote internal branches for which the likelihood confidence intervals are significantly positive ($P < 0.01$) and exclude zero. The arrow denotes a 9-bp-deletion in the control region sequence which is shared by the Bermin species and *T. guineensis* 'Cross', *T. kottae* and the Ejagham species. In addition, the species of Bermin share an amino-acid substitution at position 32 of cytochrome *b* which was not found in the other species analysed nor in any other cytochrome *b* sequence determined so far.

METHODS. Fishes were collected and stored in ethanol. DNA was extracted as described in ref. 35. Extractions with no tissue present were done to control for contamination by extraneous DNA. Amplifications were performed as in ref. 36. Primers used for cytochrome *b* were L14724 (ref. 7), H15149 (ref. 37), and for the control region: (Uli71L) 5'-TACCCCTAGCTCCCAAGCT-3' and (Uli70H) 5'-TGGTGGGCTCTTATC-ACATT-3'. The double-stranded amplification product was sequenced as in ref. 38. Sequences were analysed with the PHYLIP package³⁹. The consensus of 1,000 minimum mutation trees constructed from bootstrap replications⁴⁰ of the alignable parts of the sequences are shown. Transitions and transversions were weighted equally. Numbers refer to the frequency with which the respective branches occurred among the bootstrap



replications (alignments available from U.K.S.). When maximum likelihood trees for the same sequences were constructed, the only topological changes from the minimum mutation trees were three internal branches in the Barombi Mbo group.



are likewise closely related to one another and the subgenus *Coptodon*. Thus, in contrast to previous assumptions¹⁸, the tree suggests that the species flocks of both lakes are monophyletic.

In addition, about 350 base pairs of the rapidly evolving mitochondrial control region were sequenced from all crater lake species as well as all available members of the *S. galilaeus* and *Tilapia* (*Coptodon*) groups. The control region data were included in the analysis and the most parsimonious trees reconstructed (Fig. 2b and c). The topologies of these trees are nearly identical to the one based on cytochrome *b* alone (Fig. 2a) and show the species of Barombi Mbo as well as Bermin to be monophyletic with respect to the river species. For Barombi Mbo, bootstrap replications as well as likelihood estimates support the monophyly of the species flock. The phylogenetic results agree with the generic designations introduced by Trewavas¹⁸, except that *Pungu maclareni* was assumed¹⁸ to be derived from a distantly related tilapiine lineage^{18,22}. For Bermin, *T. (Coptodon) zillii* and *T. (C.) guineensis* 'Ivory Coast' are excluded as sister taxa of the species flock by the tree analysis as well as by the occurrence of a deletion shared by the Bermin taxa and *T. (Coptodon) guineensis* 'Cross' and *T. (C.) kottae*. The identification of *T. (Coptodon) guineensis* 'Cross' as the sister taxon to the species flock is suggested by bootstrap as well as maximum likelihood analyses of the complete data set and agrees with expectations from biogeography (Fig. 1). On the other hand, *T. (C.) kottae* and the *Coptodon*-related cichlids of Lake Ejagham could not be excluded as sister-taxa. The monophyly of the Bermin lake flock is further supported by the presence of glycine at position 32 in the cytochrome *b* sequence, where all other species have an asparagine residue. This unusual amino-acid replacement, which involves a change in charge, has thus become fixed in the Bermin species flock, presumably in the small population that colonized the lake and which subsequently gave rise to the species radiation.

The molecular data show that the species flocks in each lake are monophyletic, and suggest that they evolved within each lake after a single colonization event. An alternative explanation—allopatric origin for all 20 species—implies the independent colonization of the lakes with subsequent extinction of the source river populations—an extremely unlikely scenario. The results might also be explained by the colonization of the lakes by several species, each with different mitochondrial (mt) DNA types. If these species could hybridize, selection for and fixation of a particular type in each lake is conceivable. We find this explanation unlikely for two reasons. First, that the same events would

have occurred independently in each lake seems implausible. Second, the mtDNA types cluster in the phylogenetic trees according to the ecological characteristics of the species concerned.

There are good reasons to assume that any cichlid population in these lakes lives in sympatry *sensu ref. 1*, that is that individuals of the populations are physically capable of encountering one another with at least moderately high frequency. According to this definition, a population is sympatric even if individuals are ecologically segregated, as long as a fairly high proportion of them encounters the others along ecotones. Cichlid populations within the lakes live in sympatry, because, first, the lakes are too small to restrict gene flow to certain areas among highly mobile organisms such as tilapiine cichlids. Second, the habitats along the shorelines are uniform and free of physical barriers (Fig. 1b)^{18,19}. Therefore no microgeographical barriers exist within the lakes which would have allowed separation of microallopatric subpopulations. Third, the calderas have a uniformly conical shape so that past lake-level fluctuations could not have produced separate basins. Fourth, the lakes are isolated from the surrounding river systems by crater rims, penetrated only by small creeks. Gene flow from outside the lake is thus restricted. The isolated nature of many crater lakes in Cameroon is impressively illustrated by the complete absence of fish in some lakes located at similar altitude as Barombi Mbo and Bermin, such as Lake Benakuma²³. Finally, the species that are either benthically or pelagically oriented in their feeding behaviour both breed close to the lake bottoms. Therefore, even if there were microgeographical sorting of subpopulations based on feeding behaviour, mating would still take place in sympatry.

Most models for sympatric speciation^{6,24–30} assume a genetically based ecological diversification as a prerequisite for the evolution of increased assortative mating among different ecotypes leading to reproductive isolation. Interestingly, the basal branches of the phylogenetic trees cluster ecological groups within the lake flocks. In Bermin, the two basal lineages of the flock separate the pelagic planktivorous species from the substrate oriented feeders. In Barombi Mbo, the sequence analysis identifies three ecological groups, one containing the predatory genus *Stomatepia*, another the fine-particle feeders of the genera *Sarotherodon* and *Myaka*, and a third specialists of the genera *Konia* and *Pungu*, the latter being a sponge-feeder, for example. Thus, ecological diversification may have been a key factor responsible for speciation after colonization. □

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1. Futuyma, D. J. & Mayer, G. C. *Syst. Zool.* **29**, 254–271 (1980).
2. Grant, P. R. & Grant, B. R. in *Speciation and its Consequences* (eds Otte, D. & Endler, J. A.) 433–457 (Sinauer, Sunderland, MA, 1989).
3. Felsenstein, J. *Evolution* **35**, 124–138 (1981).
4. Coyne, J. A. *Nature* **355**, 511–515 (1992).
5. Otte, D. & Endler, J. A. (eds) *Speciation and its Consequences* (Sinauer, Sunderland, MA, 1989).
6. Kondrashov, A. S. & Mina, V. M. *Biol. J. Linn. Soc.* **27**, 201–223 (1986).
7. Meyer, A., Kocher, T. D., Basasibwaki, P. & Wilson, A. C. *Nature* **347**, 550–553 (1990).
8. Nishida, M. *Experientia* **47**, 974–979 (1991).
9. Livingstone, D. A. *Rev. Ecol. Syst.* **6**, 249–280 (1975).
10. Owen, R. B. et al. *Proc. R. Soc. B* **240**, 519–553 (1990).
11. Tiercelin, J. J. & Mondegue, A. in *Lake Tanganyika and its Life* (ed. Coulter, G. W.) 7–48 (Oxford Univ. Press, London, 1991).
12. Ribbink, A. J., March, B. A., Marsh, A. C., Ribbink, B. J. & Sharp, B. J. S. *Afr. J. Zool.* **18**, 1–180 (1983).
13. Rice, W. R. & Salt, G. W. *Evolution* **44**, 1140–1152 (1990).
14. Mayr, E. in *Evolution of Fish Species Flocks* (eds Echelle, A. A. & Kornfield, I.) 3–11 (Univ. of Maine Press, Orono, 1984).
15. McKaye, K. R., Kocher, T., Reinthal, P. & Kornfield, I. *Zool. J. Linn. Soc.* **76**, 91–96 (1982).
16. McKaye, K. R., Kocher, T., Reinthal, P., Harrison, R. & Kornfield, I. *Evolution* **38**, 215–219 (1984).
17. Sturmbauer, C. & Meyer, A. *Nature* **358**, 578–581 (1992).
18. Trewavas, E., Green, J. & Corbet, S. A. *J. Zool., Lond.* **167**, 41–95 (1972).
19. Stiassny, M., Schlieven, U. K. & Dominey, W. *Ichthyol. exp. Freshwaters* **3**, 311–346 (1992).
20. Dominey, W. & Snyder, A. M. *Env. Biol. Fish.* **22**, 155–160 (1988).
21. Greenwood, P. H. *Bull. Brit. Mus. Nat. Hist. (Zool.)* **53**, 139–203 (1987).
22. Thys van den Audenaerde, D. F. *Rev. Zool. Bot. Afr.* **82**, 285–300 (1970).

23. Kling, G. W. thesis, Duke Univ., Princeton (1987).
24. Maynard Smith, J. *Am. Nat.* **100**, 637–650 (1966).
25. Pimm, W. L. *J. Linn. Soc.* **11**, 131–139 (1979).
26. Seger, J. in *Evolution, Essays in Honour of John Maynard Smith* (eds Greenwood, P. J., Harvey, P. H. & Slatkin, M.) 43–53 (Cambridge Univ. Press, UK, 1985).
27. Udovic, D. *Am. Nat.* **116**, 621–641 (1980).
28. Rice, W. R. *Evol. Ecol.* **1**, 301–314 (1987).
29. McKaye, K. R. *Env. Biol. Fish.* **5**, 75–78 (1980).
30. Meyer, A. *Biol. J. Linn. Soc.* **39**, 279–299 (1990).
31. Kling, G. W. *Limnol. Oceanogr.* **33**, 27–40 (1988).
32. Hassert, K. Z. *Ges. Erdk. Berl.* **1912**, 7–41, 135–144, 203–216 (1912).
33. Trewavas, E. *Bull. Brit. Mus. Nat. Hist. (Zool.)* **26**, 331–419 (1984).
34. Teugels, G., Reid, G. & King, R. K. *Ann. Mus. Roy. Afr. Centr., Sci. Zool.* **266**, 1–132 (1992).
35. Pääbo, S., Gifford, J. A. & Wilson, A. C. *Nucleic Acids Res.* **16**, 9775–9787 (1988).
36. Thomas, R. H., Schaffner, W., Wilson, A. C. & Pääbo, S. *Nature* **340**, 465–467 (1989).
37. Kocher, T. D. et al. *Proc. natn. Acad. Sci. U.S.A.* **86**, 6196–6200 (1989).
38. Bachmann, B., Lücke, W. & Hunsmann, G. *Nucl. Acids Res.* **5**, 1309 (1990).
39. Felsenstein, J. P. *Version 3.3* (Univ. Washington, Seattle, 1990).
40. Felsenstein, J. *Evolution* **39**, 783–791 (1985).

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Brain regions associated with acquisition and retrieval of verbal episodic memory

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It is widely held that conscious recall of past experiences involves a specific system—episodic memory¹. Patients with amnesia have gross impairments of episodic memory while other kinds of memory remain intact^{2,3}, suggesting that a separable brain system underlies episodic memory. We have used positron emission tomography (PET) to identify components of this system in normal volunteers. A dual-task interference paradigm⁴ was used to isolate brain areas associated with acquisition, and a cueing paradigm⁵ to isolate the areas concerned with retrieval from verbal episodic memory. Acquisition was associated with activity in the left prefrontal cortex and the retrosplenial area, whereas retrieval was associated with activity in right prefrontal cortex and the precuneus. Our results provide clear evidence that episodic memory involves a network of specific prefrontal and posterior structures^{6,7} which can be fractionated into different component processes.

Brain activity was measured in six volunteers while they learned and, in another six, while they retrieved verbal material. All volunteers heard 15 paired associates, consisting of categories and relatively uncommon exemplars (such as furniture-sideboard) and were told they would have to recall them later. Recall after only one presentation was ~80% correct so that scanning during one occurrence of a list was sufficient to capture the brain activity associated with consistently successful encoding

or retrieval. During the acquisition phase, presentation of words automatically leaves legacies (priming¹) of the semantic memory¹ processes involved. These can be separated from the processes underlying episodic memory^{6,8}. Our experiments were designed to isolate the two types of encoding by subtracting a condition involving only the former processes from one involving both types. Episodic memory encoding is impaired by a difficult but structurally unrelated distractor task⁴ while automatic priming is unaffected^{8,9}. Experiment 1 (Table 1) contrasted

TABLE 1 Description of tasks

Experiment 1 (acquisition)			
	Paired-associate task	Memory performance	Distractor task*
I	Encoding †	83 ± 4%	Easy
II (control)	Passive listening ‡	—	Easy
III	Encoding †	68 ± 4%§	Difficult
IV (control)	Passive listening ‡	—	Difficult
Experiment 2 (retrieval)			
	Paired-associate task	Memory performance	
I	Recall	81 ± 5%	
II	Generation ¶	—	
III (control)	Repetition #	—	

* A joy-stick was used to move a cursor into a box in one of four different positions on a screen in a completely predictable (easy condition) or random (difficult condition) sequence. The inter-trial interval was 1 s.

† Volunteers tried to remember 15 paired associates (for example, poet-Browning) which they heard at the rate of 1 per 3 s during the scan. Immediately after the scan, volunteers filled in a mood questionnaire to prevent rehearsal of the word pairs. Recall was tested 5 min after the end of the scan.

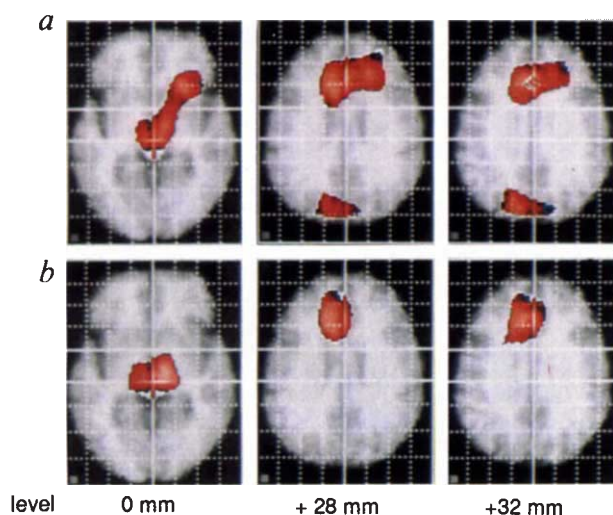
‡ Volunteers heard the same pair of words (one thousand-two thousand) 15 times at the same rate.

§ Significantly inferior to performance with easy distraction: related-*t* (d.f. = 5) = 7.3, *P* < 0.001.

|| 15 paired-associates were presented, exactly as in experiment 1, 5 min before the scan. During the scan, volunteers were presented with category labels (for example, poet) at a rate of 1 per 3 s, and had to supply the appropriate, specific exemplar (such as Browning, or the word 'pass').

¶ As in the recall condition, except that new categories were used that had not been presented previously, and the instructions were to generate any appropriate exemplar.

Subjects were presented with a (different) word to repeat every 3 s.



◀ FIG. 1 Regions significantly activated in the memory encoding condition (compared with passive listening) while performing a, the easy distracting task and b, the difficult distracting task. Areas of significant activation (*P* < 0.001) have been superimposed onto averaged magnetic resonance imaging scans (from 6 normal subjects) which have been transformed stereotactically to fit a standard atlas²⁵. Three transverse planes are shown. Levels are relative to the intercommissural plane. In both comparisons there is activation of the left superior temporal gyrus and of the left anterior cingulate cortex. Activation of these areas would be expected given the auditory word processing and attentional demands of the tasks^{26,27}. The left medial frontal gyrus (not visible in these slices; Table 2), activated primarily during difficult distraction, may be associated with the additional processes involved in performing two tasks at the same time. Left dorsolateral prefrontal cortex and retrosplenial cortex were activated only during easy distraction. A total of 12 right-handed male volunteers (6 in each experiment), who gave written informed consent, took part in the study, which was approved by the local hospital ethics committee and ARSAC (UK). Scans were obtained using a CTI model 953 B-PET scanner (CTI Inc, Knoxville, USA) with collimating septa retracted. Volunteers received a 20-s intravenous bolus of H₂¹⁵O at a concentration of 55 MBq ml⁻¹ and a flow rate of 10 ml min⁻¹ through a forearm cannula. Images were examined in ANALYZE (BRU, Mayo Foundation, Rochester, MN, USA)²⁸. Statistical analysis was performed in PROMATLAB (Math Works, Natick, MA, USA) using statistical parametric mapping (MRC Cyclotron Unit). The original brain images were transformed²⁹ into a standard stereotactic anatomical space²⁵. Resultant images extended from -20 mm below to 60 mm above the intercommissural plane. Global differences in cerebral blood flow were covaried out and significant differences between conditions were then estimated on a pixel-by-pixel basis using the *t* statistic³⁰.

TABLE 2 Activation foci in the different conditions

Acquisition (experiment 1)								
	Easy distractor				Difficult distractor			
	x (mm)	y (mm)	z (mm)	Z value	x (mm)	y (mm)	z (mm)	Z value
L. sup. temp. (22)	-56	0	-4	5.0	-54	-6	0	5.2
R. sup. temp. (21)	—	—	—	—	48	4	-8	4.1
L. ant. cing. (32)	-2	28	28	4.0	-4	22	28	5.4
Med. frontal (9/10)	—	—	—	—	-22	36	20	4.0
L. Prefrontal (46)*	-31	34	8	4.1	—	—	—	—
Retrosplenial (31/23)*	-2	-62	12	4.1	—	—	—	—
Retrieval (experiment 2)								
	Episodic				Semantic			
	x (mm)	y (mm)	z (mm)	Z value	x (mm)	y (mm)	z (mm)	Z value
L. ant. cing. (32)	-2	18	36	9.4	-2	20	36	6.9
R. thalamus	2	-22	0	7.5	6	-20	0	6.4
L. thalamus	-2	-22	8	5.1	-8	-24	12	4.1
R. frontal (47)*	26	18	0	5.5	—	—	—	—
R. frontal (10/46)*	18	28	24	4.4	—	—	—	—
L. precuneus (31)*	-6	-68	36	6.1	—	—	—	—
R. precuneus (31)*	12	-72	28	4.0	—	—	—	—

In both experiments 12 PET scans were collected and divided into blocks of 4 or 3 in which each condition was represented. To emphasize episodic memory as opposed to priming, low-frequency category-exemplar pairs were used and explicit memory instructions given in conditions 1.I, 1.III and 2.I of Table 1^{2,6}. Coordinates refer to the location, in the stereotactic space defined in ref. 25, of the maximal activity indicated by the highest Z score in a particular cerebral structure. Numbers in parentheses refer to Brodmann areas which are given to help localization of activity rather than to imply any cytoarchitectonic correlates.

* Areas where direct comparison revealed significant differences between the two conditions. Abbreviations: L, left; R, right; sup., superior; temp., temporal; ant., anterior; cing., cingulate; med., medial.

the effects of easy and difficult distractor tasks on the acquisition stage of memory. During easy distraction, brain activity associated with both episodic encoding processes and ones leading to priming in semantic memory will be observed. During difficult distraction only semantic processes occur. Thus, comparison of the two conditions identified brain activity associated with episodic encoding.

During retrieval, a task involving production of primed responses does not give any region of greater neural activation than an equivalent semantic memory task requiring only unprimed responses⁵. So experiment 2 (Table 1), which is concerned with episodic memory retrieval, also included a directly comparable task requiring only semantic memory retrieval (generation from a category such as furniture-table). Differences in activation should identify regions specifically associated with retrieval from episodic memory. Both experiments included control tasks designed to engage the non-semantic input and output demands of the experimental tasks (Table 1).

In the acquisition experiment, brain regions that were significantly more activated (showing greater regional cerebral blood flow) during encoding than during a passive listening task are shown in Figs 1a (easy distractor condition), b (difficult distractor condition) and Table 2. After difficult distraction, recall of the words was significantly reduced from 83 to 68%. Two brain areas were identified where there was marked activity during easy distraction (Fig. 1a), but no detectable activity during difficult distraction (Fig. 1b). From the logic of the experimental design, these two areas—left dorsolateral prefrontal and retrosplenial cortex—are specifically associated with episodic encoding processes. Figure 2a shows the regions in the retrieval experiment activated more by the recall task than a word repetition task; Fig. 2b shows the corresponding regions for the generation task. From the logic of the experiment, the two regions shown in Fig. 2a but not b—bilateral precuneus and right prefrontal cortex—are specifically associated with episodic memory retrieval (see also Table 2).

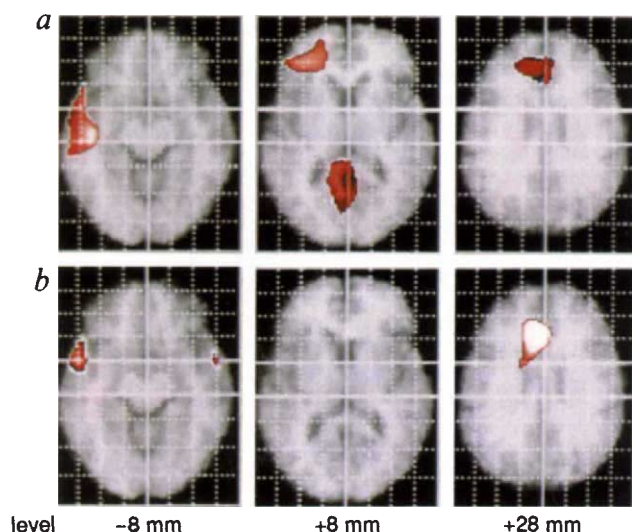


FIG. 2 Data presented as in Fig. 1. a, Regions significantly activated in the episodic memory retrieval condition (compared with word repetition). b, Regions significantly activated in the semantic memory condition. In both comparisons there is activation of the left anterior cingulate cortex, generally assumed to be associated with attention²⁷, and of the thalamus (bilaterally), long held to be implicated in memory functions². In addition, relative deactivations (not shown) were observed for both comparisons in the superior and middle temporal lobes. Similar relative deactivations have been observed in a word-list recall study and in a verbal fluency task^{10,12}. In such tasks attention is less directed towards auditory input processing than it is in the word repetition tasks with which they were compared. Activation of the precuneus bilaterally and right dorsolateral frontal cortex were observed only during retrieval from episodic memory.

The first important aspect of our results is that the same set of four regions were activated as in the only other study attempting to differentiate episodic memory from other memory processes, although in that study, acquisition and retrieval were conflated¹⁰. Particularly striking is the fact that not only were the tasks used in the earlier study very different—recall of supraspan and subspan word lists—but so also was the process contrasting with verbal episodic memory: auditory-verbal short-term memory rather than semantic memory. This convergence of evidence supports our claim that these four regions are involved in verbal episodic memory. Our observation that left frontal activity is associated with acquisition and right frontal activity with retrieval is also confirmed in other PET studies (E. Tulving *et al.*, personal communication).

The second important aspect is that the use of functional imaging has split these four regions into two involved with encoding and two with retrieval processes. The finding that the left dorsolateral prefrontal region is associated with encoding clarifies this important but poorly understood process¹. This region is held to play a major role in the executive component of working memory^{7,11,12} and is involved in the organization of supervisory thought processes^{13,14}. The major reciprocal connections between the dorsolateral prefrontal region and the hippocampus involve the retrosplenial region¹⁵, the other area activated in this study. Lesions to this region can produce amnesia^{16,17}. These observations fit with the idea that encoding involves left frontal control of hippocampal function. For retrieval, the involvement of the right prefrontal area confirms the results of an earlier PET study of memory retrieval⁵. Furthermore, lesions producing the confabulatory disorders, observable after anterior communicating artery aneurysms¹⁸ and frontal head injury¹⁹, involve similar areas of prefrontal cortex and occur more frequently after right- rather than left-sided damage²⁰. Memory monitoring and verification are likely to be the critical processes impaired¹⁴. The involvement of the right frontal lobe is of special interest in that our task and that used in the previous PET study of memory retrieval⁵ tested only verbal memory. Less clear is the function of the other area involved, the precuneus. Little is known about this structure from lesion studies. One possibility is that it is concerned with the retrieval of visual images^{10,21}. This would be compatible with the known posterior cortical localization of visual imagery²².

In common with nearly all relevant functional imaging studies^{5,10}, our study has failed to show selective activation of the medial brain structures (apart from the thalamus), damage to which causes amnesia². Of these structures, the mamillary bodies may be too small to resolve. But the lack of selective activation of the hippocampus must be theoretically important²³. Perhaps memory encoding in the hippocampus involves only very sparse neuronal activation²⁴. Nevertheless, our study reveals a network of prefrontal and posterior cortical structures^{6,7}, with distinct roles in the different components of episodic memory. □

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1. Tulving, E. *Elements of Episodic Memory* (Oxford Univ. Press, UK, 1983).
2. Mayes, A. R. *Human Organic Memory Disorders* (Cambridge Univ. Press, UK, 1988).
3. Baddeley, A. *Human Memory* (Erlbaum, Hove, 1990).
4. Baddeley, A. D., Eldridge, M., Lewis, V. & Thompson, N. J. *exp. Psychol. Gen.* **113**, 518–540 (1984).
5. Squire, L. R. *et al. Proc. natn. Acad. Sci. U.S.A.* **89**, 1837–1841 (1992).
6. Warrington, E. K. & Weiskrantz, L. *Neuropsychologia* **20**, 233–248 (1982).
7. Goldman-Rakic, P. S. *Rev. Neurosci.* **11**, 137–156 (1988).
8. Jacoby, L. J., Ste-Marie, D. & Toth, J. P. *Attention: Selection, Awareness and Control* (eds Baddeley, A. & Weiskrantz, L.) (Oxford Univ. Press, UK, 1993).
9. Parkin, A. J., Reid, T. K. & Russo, R. *Mem. Cog.* **18**, 507–514 (1990).
10. Grasby, P. M. *et al. Brain* **116**, 1–20 (1993).
11. Petrides, M., Alivisatos, B., Meyer, E. & Evans, A. *Proc. natn. Acad. Sci. U.S.A.* **90**, 878–882 (1993).
12. Frith, C. D., Friston, K. J., Liddle, P. F. & Frackowiak, R. S. J. *Proc. R. Soc. Lond.* **B244**, 241–246 (1991).
13. Baddeley, A. D. *Working Memory* (Oxford Univ. Press, UK, 1986).
14. Shallice, T. *From Neuropsychology to Mental Structure* (Cambridge Univ. Press, UK, 1988).
15. Goldman-Rakic, P. S., Selemon, L. D. & Schwartz, M. S. *Neuroscience* **12**, 719–743 (1984).
16. Valenstein, E. *et al. Brain* **110**, 1631–1646 (1987).
17. Rudge, P. & Warrington, E. K. *Brain* **114**, 349–360 (1991).

18. Stuss, D. T. *et al. Neurology*, **28**, 1166–1172 (1978).
19. Baddeley, A. D. & Wilson, B. *Autobiographical Memory* (ed. Rubin, D.) (Cambridge Univ. Press, New York, 1986).
20. Burgess, P. W. thesis, Univ. London (1992).
21. Roland, P. E. & Seitz, R. J. in *Visualization of Brain Functions in the Human Brain* (eds Ottoson, D. & Rostene, W.) 141–151 (Stockton, London, 1989).
22. Farah, M. J. *Cognition* **18**, 245–272 (1984).
23. Squire, L. R. *Psychol. Rev.* **99**, 195–231 (1992).
24. Rolls, E. T. & Treves, A. *Network* **1**, 407–421 (1990).
25. Talairach, J. & Tournoux, P. *Co-Planar Stereotactic Atlas of the Human Brain* (Thieme, Stuttgart, 1988).
26. Wise, R. *et al. Brain* **114**, 1803–1817 (1991).
27. Posner, M. I. & Petersen, S. E. *A. Rev. Neurosci.* **13**, 25–42 (1990).
28. Robb, R. A. & Hanson, D. P. *Aust. Phys. Eng. Sci. Med.* **14**, 9–30 (1991).
29. Friston, K. J., Frith, C. D., Liddle, P. F. & Frackowiak, R. S. J. *J. comp. Ass. Tom.* **15**, 634–639 (1991).
30. Friston, K. J., Frith, C. D., Liddle, P. F. & Frackowiak, R. S. J. *J. cereb. Blood Flow Metab.* **11**, 690–699 (1991).

Role of guanylyl cyclase and cGMP-dependent protein kinase in long-term potentiation

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SEVERAL lines of evidence suggest that cyclic GMP might be involved in long-term potentiation (LTP) in the hippocampus^{1–6}. Arachidonic acid, nitric oxide and carbon monoxide, three molecules that have been proposed to act as retrograde messengers in LTP^{7–9}, all activate soluble guanylyl cyclase^{1,10,11}. We report here that an inhibitor of guanylyl cyclase blocks the induction of LTP in the CA1 region of hippocampal slices. Conversely, cGMP analogues produce long-lasting enhancement of the excitatory postsynaptic potential if they are applied at the same time as weak tetanic stimulation of the presynaptic fibres. The enhancement is spatially restricted, is not blocked by valeric acid (APV), nifedipine, or picrotoxin, and partially occludes LTP. This synaptic enhancement may be mediated by the cGMP-dependent protein kinase (PKG). Inhibitors of PKG block the induction of LTP, and activators of PKG produce activity-dependent long-lasting enhancement. These results suggest that guanylyl cyclase and PKG contribute to LTP, possibly as activity-dependent presynaptic effectors of retrograde messengers.

An inhibitor of soluble guanylyl cyclase LY83583 (50% inhibitory concentration IC₅₀ = 2 μM)¹², completely blocked the induction of LTP at a concentration of 5 μM (Fig. 1a), and occasionally blocked it at a concentration of 0.5 μM (two of six experiments). The effects of the inhibitor are relatively specific. LY83583 (5 μM) did not affect either the maintenance of LTP (Fig. 1a), the baseline excitatory postsynaptic potential (e.p.s.p.; 96.1 ± 6.6%, *n* = 9), or the NMDA (*N*-methyl-D-aspartate) component of e.p.s.p. measured in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), which blocks the non-NMDA component (102 ± 14%, *n* = 3).

Conversely, application of the membrane-permeable analogue 8-Br-cGMP (100 μM) at the same time as weak tetanic stimulation (50 Hz, 0.5 s) ('paired' training) produced an immediate potentiation of the e.p.s.p. that lasted for at least 1 h (Fig. 1c). 8-Br-cGMP paired with weak stimulation produced significant enhancement even in the presence of 5 μM LY83583 (160.2 ± 5.9%, *n* = 5, *t*[4] = 10.3, *P* < 0.01, comparing the mean

FIG. 1 Inhibition of LTP in the CA1 region of hippocampus by the guanylyl cyclase (GC) inhibitor LY83583, and long-term enhancement by 8-Br-cGMP. **a**, Average potentiation of the e.p.s.p. by strong tetanus (twice at 100 Hz for 1 s each separated by 20 s, filled triangles) in slices pretreated with artificial cerebrospinal fluid (ACSF) containing 5 μ M LY83583 (filled squares, $91.1 \pm 11.5\%$ s.e.m., $n = 5$, $t[4] = 0.77$, NS (not significant) comparing the mean potential 50 to 60 min post-tetanus to the average for 30 min pre-tetanus), and in slices in which perfusion with LY83583 began 10 min after tetanus (squares, $166.0 \pm 17.6\%$, $n = 5$, $t[4] = 3.75$, $P < 0.05$). Washout with ACSF started after 30 min of drug application. LTP was significantly reduced in slices pretreated with 5 μ M LY83583, compared with slices in which the inhibitor was applied during the maintenance of LTP ($t[8] = 3.56$, $P < 0.01$). Inset, Representative records of the e.p.s.p. before and 50 to 60 min after tetanus in a slice pretreated with 5 μ M LY83583 ('induction') and in a slice in which perfusion with 5 μ M LY83583 began 10 min after tetanus ('maintenance'). **b**, 8-Br-cGMP alone does not produce long-term enhancement (100 μ M, applied during the horizontal bar, $n = 4$). **c**, Long-term enhancement by paired training, in which a weak tetanus (single filled triangle) was given during the perfusion of 8-Br-cGMP ($178.5 \pm 16.6\%$, $n = 12$; $t[11] = 4.73$, $P < 0.01$). **d**, Unpaired training in which the weak tetanus occurred 5 min before the 8-Br-cGMP perfusion ($n = 5$). Paired training produced significantly greater enhancement than 8-Br-cGMP alone, weak stimulation alone, or unpaired training ($P < 0.05$ in each case, Duncan's multiple range test). The average prevalues were **a**, -0.34 ± 0.04 mV ms^{-1} (maintenance) and -0.25 ± 0.04 (induction); **b**, -0.29 ± 0.03 ; **c**, -0.27 ± 0.03 ; and **d**, -0.24 ± 0.02 . **METHODS**. Adult male guinea-pigs were housed and killed in accordance with the guidelines of the Health Sciences Division of Columbia University. Transverse slices (400 μ m) of hippocampus were rapidly prepared and maintained in an interface chamber at 30 $^{\circ}\text{C}$, where they were subsumed with saline (ACSF) consisting of (in mM): 124 NaCl, 4.4 KCl, 2.0 CaCl_2 , 1.0 Na_2HPO_4 , 2.0 MgSO_4 ,

25 NaHCO_3 , 10 glucose, bubbled with 95% O_2 , 5% CO_2 . A bipolar tungsten stimulating electrode was placed in the middle of the stratum radiatum in the CA1 region and extracellular field potentials were recorded using a glass microelectrode (3–11 Mohm, filled with ACSF) also in the stratum radiatum. The stimulation intensity was adjusted to give field e.p.s.p. amplitudes of ~ 1.0 mV, so that the weak tetanus would produce decrementing potentiation, and test responses were elicited at 0.02 Hz. LY83583 and KT5823 were dissolved in dimethyl sulphoxide (DMSO) immediately before the experiment and diluted to the desired concentration in ACSF. The final concentration of DMSO was 0.05% or less.

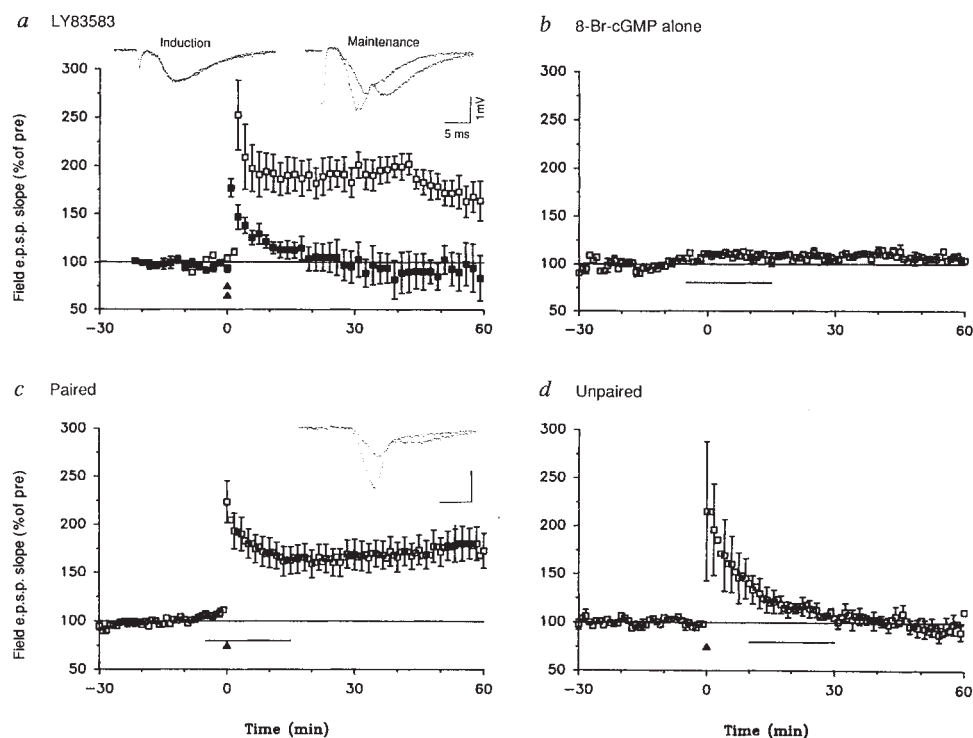
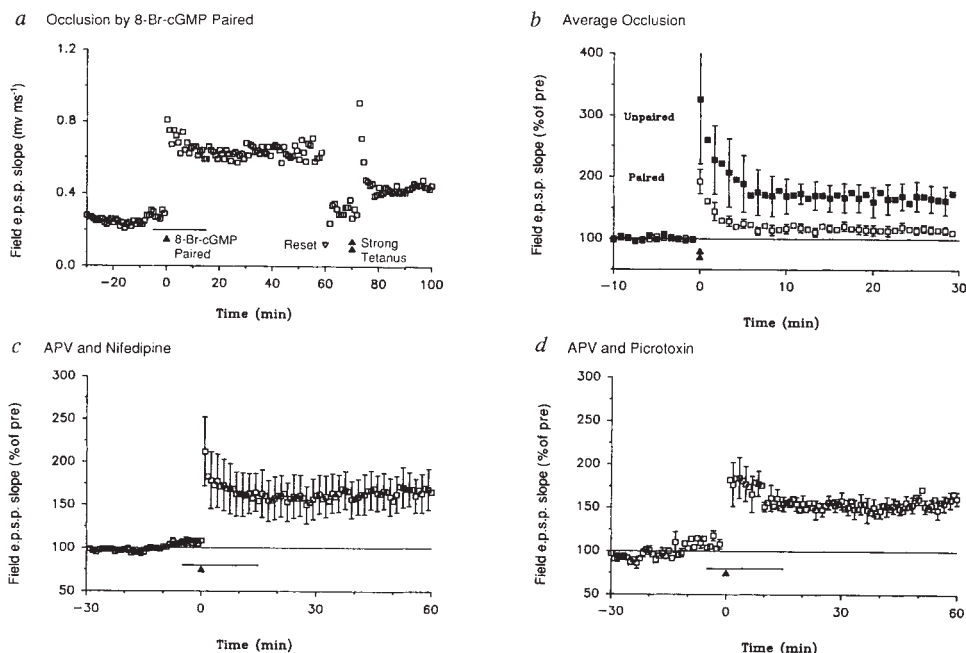


FIG. 2 Activity-dependent long-term enhancement by 8-Br-cGMP partially occludes tetanic LTP and is not blocked by APV, nifedipine or picrotoxin. **a**, Example of modest potentiation produced by a strong tetanus following long-term enhancement by paired 8-Br-cGMP (filled triangle and bar). At the inverted triangle the test intensity was decreased to return the field e.p.s.p. slope to about its original value. **b**, Average potentiation by a strong tetanus following paired 8-Br-cGMP training in seven experiments like the one shown in **a** (squares, $115.1 \pm 7.0\%$). In three of these experiments, the test intensity was reduced before the strong tetanus, as shown in **a**. In the remaining four it was not. The results were similar in the two cases and have been pooled. In other experiments (filled squares), significant potentiation was produced by the strong tetanus when it was preceded by unpaired 8-Br-cGMP ($166.9 \pm 23.0\%$, $n = 4$; $t[9] = 2.69$, $P < 0.05$ compared to unpaired training). **c**, The effect of paired training was not blocked by 50 μ M APV ($n = 6$), or by 50 μ M APV and 10 μ M nifedipine ($n = 5$). Results with and without nifedipine were not significantly different and have been pooled ($167.3 \pm 22.1\%$, $t[10] = 3.05$, $P < 0.05$). **d**, Paired training with 50 μ M APV and 100 μ M picrotoxin present throughout the experiment ($158.0 \pm 6.2\%$, $n = 4$; $t[3] = 9.41$, $P < 0.01$). In experiments with picrotoxin, the concentrations of CaCl_2 and MgSO_4 in the saline were raised to 4.0 mM, and the CA3 region was removed to prevent epileptiform activity. The enhancement produced by 8-Br-cGMP paired with weak stimulation in normal saline, in the



presence of APV, APV and nifedipine, or APV and picrotoxin were not significantly different (Duncan's multiple range test). Average prevalues in **c** and **d** were both -0.28 ± 0.03 mV ms^{-1} .

e.p.s.p. 50 to 60 min post-tetanus to the average for 30 min pre-tetanus), suggesting that LY83583 does in fact act on the cyclase and not on the steps downstream from it. The effects of 8-Br-cGMP and weak stimulation were synergistic. Neither 8-Br-cGMP alone (Fig. 1b) nor weak stimulation alone ($91.1 \pm 6.5\%$, $n=6$) produced any significant enhancement. Furthermore, application of 8-Br-cGMP 5 min after weak stimulation ('unpaired' training) did not produce significant enhancement (Fig. 1d).

The long-term enhancement produced by 8-Br-cGMP was restricted to the active presynaptic pathway in experiments in which two pathways were tested in the same slice. When 8-Br-cGMP was paired with weak stimulation of one pathway, the e.p.s.p. in that pathway was rapidly enhanced and remained enhanced for at least 1 h ($172.5 \pm 12.7\%$, $n=5$, $t[4]=5.71$, $P<0.01$). By contrast, the e.p.s.p. in the second, independent pathway, which received unpaired training, showed no significant long-term effect ($107.4 \pm 9.4\%$).

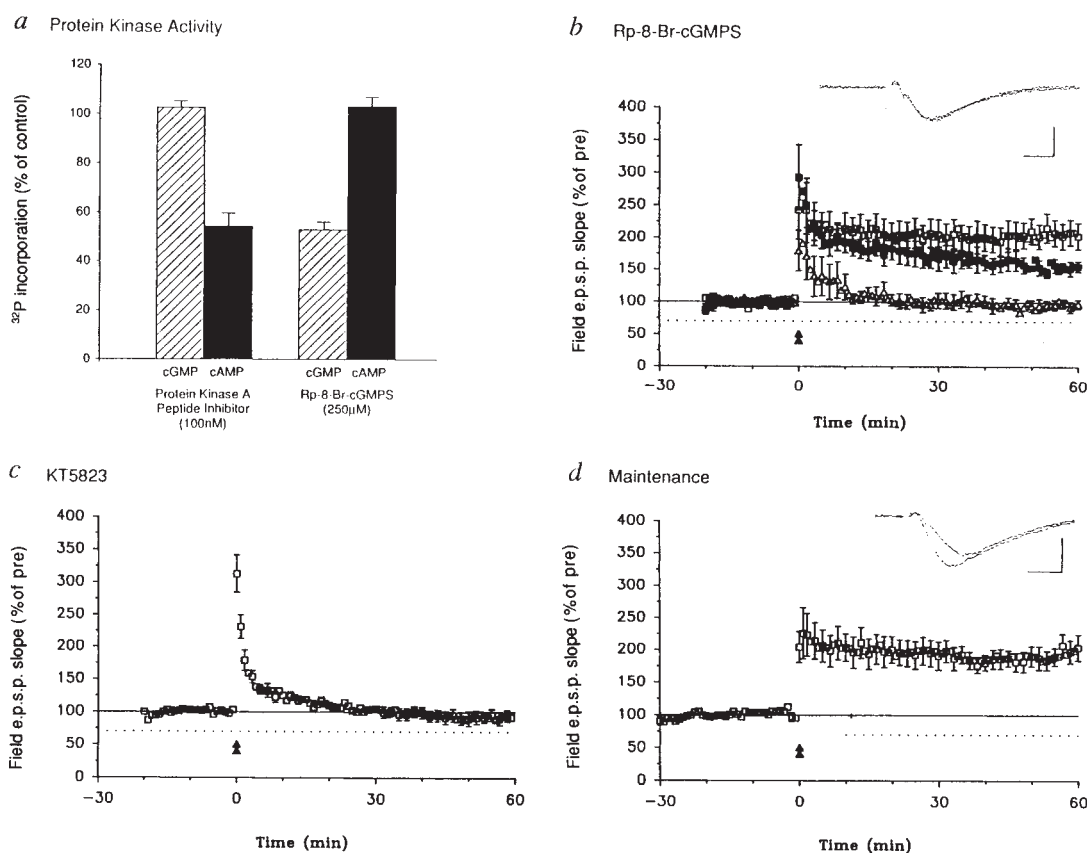
This activity-dependent long-term enhancement was also obtained with another cGMP analogue, dibutyl cGMP (250 μ M, $207.1 \pm 52.4\%$, $n=3$), as well as with the acetoxymethyl ester of cGMP (cGMP/AM), a new and highly potent derivative that crosses membranes readily, and hydrolyses intracellularly

to release cGMP itself as the active species¹³ (1 μ M, $201.1 \pm 18.7\%$, $n=5$, $t[4]=5.42$, $P<0.01$). Because cGMP, unlike 8-Br-cGMP or dibutyl cGMP, is rapidly degraded by phosphodiesterases, this result indicates that a transient increase of intracellular cGMP levels is sufficient for long-lasting enhancement by paired training. By contrast, the e.p.s.p. was not potentiated by atrial natriuretic peptide, which stimulates the membrane-bound form of guanylyl cyclase in the hippocampus^{2,14}, paired with weak stimulation (1 μ M, $114.0 \pm 11.8\%$, $n=4$). This result suggests that the soluble cytoplasmic form of guanylyl cyclase may be preferentially involved in LTP. Similarly, the e.p.s.p. was not potentiated but depressed by a cAMP analogue, 8-(4-chlorophenylthio)-adenosine 3',5'-cyclic monophosphate (100 μ M), paired with weak stimulation ($48.4 \pm 13.2\%$, $n=4$, $t[3]=3.90$, $P<0.05$).

The long-term enhancement by 8-Br-cGMP paired with weak stimulation partially occluded normal LTP, suggesting that enhancement by cGMP may contribute to LTP. One hour after 8-Br-cGMP paired training, strong tetanus (100 Hz, 2×1 s) produced only modest potentiation that was not significant. As a control, strong tetanus did produce significant potentiation 1 h after 8-Br-cGMP unpaired training (Fig. 2a, b).

8-Br-cGMP does not produce its effects simply by enhancing

FIG. 3 PKG activity in hippocampus, and the effects of PKG inhibitors on the induction and maintenance of LTP. a, cAMP- and cGMP-induced phosphorylation of PKG peptide substrate (G-substrate) in hippocampal homogenate. Left, cGMP-induced phosphorylation was not affected by PKA inhibitor (100 nM, $102.4 \pm 2.7\%$, $n=3$), whereas cAMP-induced phosphorylation was significantly decreased ($54.0 \pm 5.5\%$, $n=3$; $t[2]=8.36$, $P<0.05$). Right, cAMP-induced phosphorylation was not affected by Rp-8-Br-cGMPs at doses tested (10 μ M–10 mM), whereas cGMP-induced phosphorylation was significantly decreased (250 μ M, $53.0 \pm 2.8\%$, $n=4$; $t[3]=16.79$, $P<0.01$). b, Inhibition of LTP by Rp-8-Br-cGMPs. Average potentiation by a strong tetanus in ACSF (squares, $206.7 \pm 19.9\%$, $n=9$; $t[8]=5.36$, $P<0.01$) and in ACSF containing 1 nM (filled squares, $154.4 \pm 9.3\%$, $n=5$) or 10 μ M Rp-8-Br-cGMPs (empty triangles, $95.6 \pm 7.3\%$, $n=5$). Perfusion with Rp-8-Br-cGMPs started at least 30 min before the tetanus. c, Inhibition of LTP by KT5823 (10 μ M, $92.7 \pm 9.2\%$, $n=5$). Compared to normal, LTP was significantly reduced by Rp-8-Br-cGMPs (1 nM, 100 nM, 10 μ M), Rp-cGMPs, and KT5823 (Duncan's multiple range test). d, Rp-cGMPs (10 μ M $n=5$) or KT5823 (10 μ M, $n=2$) applied 5 min after strong tetanus did not block the maintenance of LTP. Results with Rp-cGMPs and KT5823 were not significantly different, and have been pooled ($193.8 \pm 15.6\%$; $t[6]=6.01$, $P<0.01$; not significantly different from normal LTP). The average prevalues were b, -0.29 ± 0.03 mV ms⁻¹ (normal), -0.26 ± 0.02 (1 nM), and -0.22 ± 0.02 (10 μ M); c, -0.24 ± 0.02 ; and d, -0.30 ± 0.03 .



METHODS. Whole hippocampus was homogenized in buffer containing 20 mM Tris-HCl, pH 7.4, 25 mM β -mercaptoethanol, 0.25 M sucrose, and 0.025 mM PMSF. The homogenate was then centrifuged at 4 °C, 13,000g for 30 min and the supernatant was used for the protein kinase assay. The assay was done in a total volume of 20 μ l containing 25 mM Tris-HCl, pH 7.4, 6 mM MgSO₄, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, and 500 μ g ml⁻¹ PKG substrate peptide (Arg-Lys-Arg-Ser-Arg-Ala-Glu). The sequence of the PKA inhibitor is Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp. After 30 min incubation at 37 °C, the reaction mixture was applied to phosphocellulose papers and washed 3 times with phosphate buffer. The washed phosphocellulose papers were then counted in a scintillation spectro-photometer.

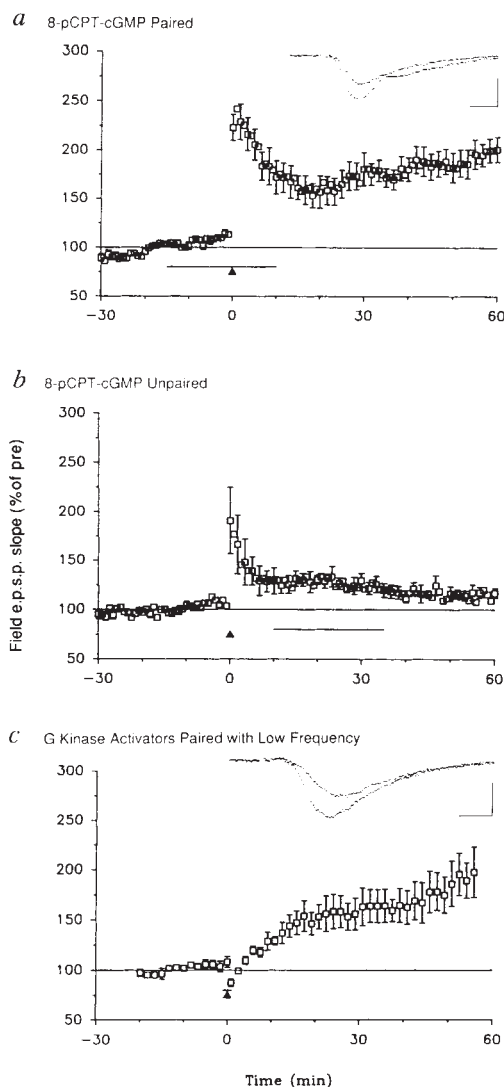


FIG. 4 Long term enhancement of synaptic transmission by PKG activators. *a*, Long-term enhancement by paired training, in which a weak tetanus occurred during the 8-pCPT-cGMP perfusion ($198.4 \pm 16.4\%$, $n=6$; $t[5]=6.00$, $P<0.01$). *b*, Decrementing potentiation by unpaired training in which the weak tetanus occurred 5 min before the 8-pCPT-cGMP perfusion ($113.9 \pm 3.6\%$, $n=5$). The enhancement produced by 8-pCPT-cGMP paired training was significantly greater than that produced by unpaired training ($t[9]=4.58$, $p<0.01$). *c*, Long-term enhancement by 8-pCPT-cGMP ($n=3$, $10 \mu\text{M}$) or β -phenyl-1, N^2 -ethenoguanosine-3',5'-cyclic monophosphate (PET-cGMP) ($n=3$, $10 \mu\text{M}$) paired with low-frequency stimulation (0.25 Hz, 100 s). Results with 8-pCPT-cGMP and PET-cGMP were not significantly different and have been pooled ($178.5 \pm 20.8\%$, $n=6$; $t[5]=3.78$, $P<0.05$). The average prevalues were *a*, $-0.23 \pm 0.04 \text{ mV ms}^{-1}$; *b*, -0.22 ± 0.02 ; and *c*, -0.30 ± 0.04 .

Ca^{2+} influx through the NMDA receptor channel or the L-type voltage-dependent Ca^{2+} channel during weak stimulation. 8-Br-cGMP paired with weak stimulation still produced significant long-term enhancement in the presence of the NMDA receptor blocker APV (D-2-amino-5-phosphonopentanoate; $50 \mu\text{M}$) and the L-type voltage-dependent Ca^{2+} channel blocker nifedipine ($10 \mu\text{M}$) (Fig. 2c). Finally, 8-Br-cGMP does not simply produce its effect by the disinhibition of inhibitory neurons, because 8-Br-cGMP paired with weak stimulation still potentiated the e.p.s.p. in the presence of APV and the GABA-receptor blocker, picrotoxin (Fig. 2d).

Among the possible candidates for cGMP-binding target proteins, one well established candidate is PKG^{15,16}. However, previous studies have not demonstrated the presence of PKG in hippocampus by immunocytochemistry, using an antibody against one isoform of the kinase¹⁷. As a more general test, we did an *in vitro* protein kinase assay by measuring phosphorylation of an exogenous PKG peptide substrate (G-substrate). Both 8-Br-cGMP and 8-Br-cAMP increased phosphorylation of the G-substrate, but specific inhibitors of PKG or protein kinase A (PKA) selectively reduced the phosphorylation induced by 8-Br-cGMP or 8-Br-cAMP (Fig. 3a). These results suggest that PKG as well as PKA is present in the hippocampus.

Pretreatment of slices with the PKG inhibitor Rp-8-Br-cGMPs blocked normal LTP at a dose of $10 \mu\text{M}$ and reduced it at 1 nM (Fig. 3b) and 100 nM ($111.9 \pm 10.2\%$, $n=5$). Rp-cGMPs ($10 \mu\text{M}$), a weaker and less permeable inhibitor, also

reduced LTP ($129.5 \pm 11.1\%$, $n=8$), and KT5823 ($10 \mu\text{M}$), a structurally different PKG inhibitor, blocked it completely (Fig. 3c). By contrast, inhibition of PKA by Rp-cAMPS only slightly decreases LTP during the first hour, but completely blocks a later phase of LTP¹⁸. Established LTP was not affected by Rp-cGMPs or KT5823 (Fig. 3d), suggesting that the inhibitors are relatively specific.

Conversely, a selective PKG activator¹⁹, 8-(4-chlorophenylthio)-cGMP 3',5'-cyclic monophosphate (8-pCPT-cGMP, $10 \mu\text{M}$) paired with weak stimulation produced a rapid and long-lasting enhancement (Fig. 4a). 8-pCPT-cGMP alone ($102.9 \pm 4.5\%$, $n=4$) or applied 5 min after weak stimulation failed to produce long-term enhancement (Fig. 4b). Subsequent strong tetanus after 8-pCPT-cGMP paired training produced no significant potentiation ($119.9 \pm 11.9\%$, $n=5$). However, strong tetanus produced significant enhancement after 8-pCPT-cGMP unpaired training as compared with either prestimulation ($159.9 \pm 9.9\%$, $n=5$, $t[4]=6.07$, $P<0.01$) or the paired training group ($t[8]=2.58$; $P<0.05$, comparing paired training to unpaired training).

Even low-frequency stimulation (0.25 Hz) can induce LTP when paired with postsynaptic depolarization or carbon monoxide^{8,20}. However, low-frequency stimulation paired with 8-Br-cGMP produced only long-lasting depression, rather than potentiation of the e.p.s.p.³⁸. By contrast, low-frequency stimulation induced long-term enhancement of the e.p.s.p. when paired with two different PKG activators (Fig. 4c). These results are

consistent with a role for PKG in LTP, and suggest that under some circumstances cGMP analogues can also activate other mechanisms that may be involved in long-term depression.

The present results suggest that soluble guanylyl cyclase and PKG are involved in the induction of LTP and could serve as target proteins for retrograde messengers. Consistent with this idea, recent studies indicate that 8-Br-cGMP increases both the amplitude of evoked excitatory postsynaptic currents (e.p.s.cs) and the frequency of miniature e.p.s.cs in hippocampal cultures²¹, suggesting that cGMP acts presynaptically. However, postsynaptic mechanisms may also be involved. cGMP and PKG have also been shown to produce activity-dependent long-lasting effects in cerebellum and sensory-motor cortex^{22–24}. Because these effects are implicated in procedural learning^{25,26}, whereas LTP in hippocampus is thought to be involved in declarative learning²⁷, it is possible that these different types of learning at the behavioural level may involve, in part, similar underlying biochemical mechanisms. □

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1. Verma, A., Hirsch, D. J., Glatt, C. E., Ronnett, G. V. & Snyder, S. H. *Science* **259**, 381–384 (1993).
2. DeVente, J. & Steinbusch, H. W. *Acta Histochem.* **92**, 13–38 (1992).
3. Haley, J. E., Wilcox, G. L. & Chapman, P. F. *Neuron* **8**, 211–216 (1992).
4. East, S. J. & Garthwaite, J. *Neurosci. Lett.* **123**, 17–19 (1991).

5. Chetkovich, D. M., Klann, E. & Sweatt, J. D. *Neuro Report* **4**, 919–922 (1993).
6. Hawkins, R. D., Zhuo, M. & Arancio, O. *J. Neurobiol.* (in press).
7. Bliss, T. V. P. & Collingridge, G. L. *Nature* **361**, 31–39 (1993).
8. Zhuo, M., Small, S. A., Kandel, E. R. & Hawkins, R. D. *Science* **260**, 1946–1950 (1993).
9. Stevens, C. F. & Wang, Y. Y. *Nature* **364**, 147–149 (1993).
10. Snider, R. M., McKinney, M., Forray, C. & Richelson, E. *Proc. natn. Acad. Sci. U.S.A.* **81**, 3905–3909 (1984).
11. Garthwaite, J., Charles, S. L. & Chess-Williams, R. *Nature* **326**, 385–388 (1988).
12. Fleisch, J. H. et al. *J. pharmac. exp. Ther.* **229**, 681–689 (1984).
13. Schultz, C. et al. *J. biol. Chem.* **268**, 6316–6322 (1993).
14. Schulz, S., Yuen, P. S. T. & Garbers, D. L. *Trends pharmac. Sci.* **12**, 116–120 (1991).
15. White, R. E. et al. *Nature* **361**, 263–266 (1993).
16. Tsou, K., Snyder, G. L. & Greengard, P. *Proc. natn. Acad. Sci. U.S.A.* **90**, 3462–3465 (1993).
17. Schlichter, D. J., Casnellie, J. E. & Greengard, P. *Nature* **273**, 61–62 (1978).
18. Frey, U., Huang, Y.-Y. & Kandel, E. R. *Science* **60**, 1661–1664 (1993).
19. Geiger, J., Nolte, D., Butt, E., Sage, S. O. & Water, U. *Proc. natn. Acad. Sci. U.S.A.* **89**, 1031–1035 (1992).
20. Wigström, H., Gustafsson, B., Huang, Y.-Y. & Abraham, W. C. *Acta physiol. scand.* **126**, 317–319 (1986).
21. Arancio, O., Kandel, E. R. & Hawkins, R. D. *Soc. Neurosci. Abstr.* **19**, 241 (1993).
22. Shibuki, K. & Okada, D. *Nature* **349**, 326–328 (1991).
23. Ito, M. & Karachot, L. *Neuroreport* **1**, 129–132 (1990).
24. Woody, C. D., Swartz, B. E. & Gruen, E. *Brain Res.* **158**, 373–395 (1978).
25. Ito, M. A. *Rev. Neurosci.* **12**, 85–102 (1989).
26. Brons, J. F. & Woody, C. D. *J. Neurophysiol.* **44**, 605–615 (1980).
27. Squire, L. R. & Zola-Morgan, S. *Science* **253**, 1380–1386 (1991).
28. Zhuo, M., Kandel, E. R. & Hawkins, R. D. *Neuro Report* (in the press).

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Limb alterations in brachypodism mice due to mutations in a new member of the TGF β -superfamily

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THE mutation *brachypodism* (*bp*) alters the length and number of bones in the limbs of mice but spares the axial skeleton^{1,2}. It illustrates the importance of specific genes in controlling the morphogenesis of individual skeletal elements in the tetrapod limb^{3,4}. We now report the isolation of three new members of the transforming growth factor- β (TGF- β) superfamily⁵ (growth/differentiation factors (GDF) 5, 6 and 7) and show by mapping, expression patterns and sequencing that mutations in *Gdf5* are responsible for skeletal alterations in *bp* mice. GDF5 and the closely related GDF6 and GDF7 define a new subgroup of factors related to known bone- and cartilage-inducing molecules, the bone morphogenetic proteins (BMPs)⁶. Studies of *Bmp5* mutations in *short ear* mice have shown that at least one other BMP gene is also required for normal skeletal development⁷. The highly specific skeletal alterations in *bp* and *short ear* mice suggest that different members of the BMP family control the formation of different morphological features in the mammalian skeleton.

The *bp* mutation acts cell non-autonomously *in vitro* and *in vivo*, and may disrupt production of a signal that stimulates mesenchyme aggregation and chondrogenesis in limbs^{8,9}. To test

whether the skeletal alterations in brachypod mice could be due to mutations in a member of the BMP family of secreted signalling molecules we determined the mouse chromosome map locations of several BMP and BMP-related genes. The previously described *Op1*/(*Bmp7*)^{10,11} and *Op2*/(*Bmp8*)¹² genes both map outside the candidate interval for the *bp* mutation, as do the genes for *Bmp2* - *Bmp6* (refs 7, 13).

Three new BMP-related factors were identified by degenerate polymerase chain reaction (PCR; Fig. 1) and designated GDF5, GDF6 and GDF7. Like other BMPs and TGF- β superfamily members, each of the new factors contains a putative polybasic proteolytic processing site followed by a carboxy-terminal region containing seven conserved cysteine residues⁵. Previously characterized BMPs fall into three homology groups^{6,12}: the BMP2/BMP4 subfamily; the *Vg1* (BMP6)/*OP1* (BMP7)/BMP5/*OP2* (BMP8) subfamily; and BMP3. Molecules within each group share 74–92% identity in the amino-acid sequence of the mature C-terminal signalling region. Members of different subfamilies share 40–60% identity. With the exception of a 26-amino-acid glycine-rich insert present in GDF7, GDF5, GDF6 and GDF7 share 80–86% identity with each other in the mature C-terminal region, and 56–57%, 50–54% and 46–47% identity respectively with members of the three BMP subfamilies mentioned. Lower identity scores were seen with other TGF- β superfamily members. These comparisons suggest that GDF5, GDF6 and GDF7 genes define a new subgroup of BMP-related factors. Chromosome mapping has shown that the *Gdf5* locus maps between the *agouti* and *Src* loci on chromosome 2 (Fig. 2). The *bp* locus has been mapped to the same region¹⁴, identifying *Gdf5* as a candidate for the gene defective in brachypod mutants. *Gdf6* and *Gdf7* map to the proximal ends of chromosomes 4 and 12 and are not obvious candidates for other known mouse mutations (data not shown).

To test whether GDF5 is the normal product of the *bp* locus, we first isolated a full-length complementary DNA clone for the wild-type transcript. A probe from the mature region of GDF5 hybridized to a single 2.5-kilobase (kb) transcript in embryonic RNA, peaking at day 12.5 of development (data not shown). The complete sequence of a 2,329-base-pair (bp) cDNA clone of the embryonic transcript is shown in Fig. 1b. The wild-type sequence contains a single large open reading frame of 495 amino acids.

We next looked for alterations of *Gdf5* sequences in three independent *bp* mutations. Two of the mutations arose on the

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A/J and BALB/cJ inbred mouse strains, which were included in all experiments as parental controls (Fig. 3 legend). Southern blot analysis did not detect major disruptions of *Gdf5* genomic sequences in any of the mutations; we therefore amplified coding sequences from RNA and analysed them by DNA sequencing. All three mutant alleles contained frameshift mutations in the *Gdf5* open reading frame (Fig. 3). In *bp/bp* mice, bases 477–483 of the wild-type sequence are deleted, followed by an inversion of bases 484–496. The resulting frameshift creates a translational stop codon 62 amino acids later. In *bp^{3j}/bp^{3j}* mice, a CG dinucleotide in the wild-type sequence is replaced by a single T at position 876. This frameshift mutation produces a translational stop at the next codon. In *bp^j/bp^j* mice, a stretch of three Gs in the wild-type sequence is replaced by four Gs in the mutant (positions 1,444–1,448). This change produces a stop codon 41

amino acids later. Neither sequence alteration found in the *bp^j* and *bp^{3j}* mutants was present in the A/J or BALB/cJ parental control strains, strongly arguing that each was the result of a new mutation at the *bp* locus.

Active signalling molecules in the TGF- β superfamily are formed from the C-terminus of a larger precursor protein⁵. The frameshift mutations in *bp*, *bp^j* and *bp^{3j}* all occur before the mature signalling portion of GDF5 and should represent functional null mutations. To compare the skeletal phenotypes of the different alleles, we prepared skeletons from mice homozygous for the *bp^j* and *bp^{3j}* mutations. As reported for the *bp* mutation on an outbred background^{1,2}, the new mutations on coisogenic backgrounds produce specific defects in limb morphology (Fig. 4a, b, and other data not shown). The long bones of the limb are slightly shorter, and the feet are much

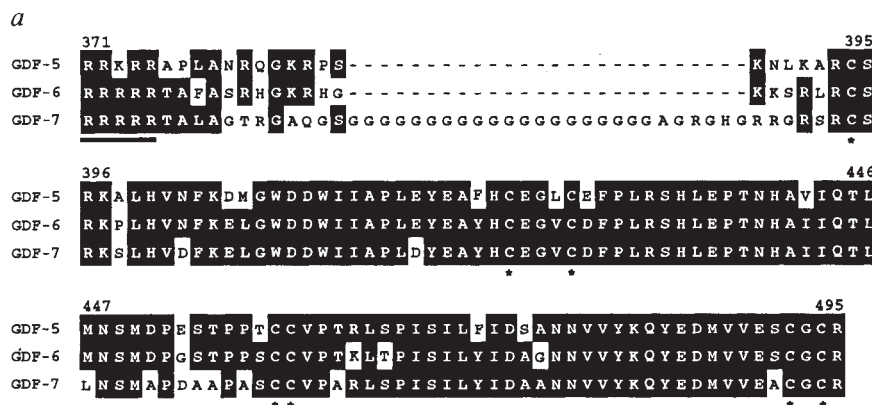
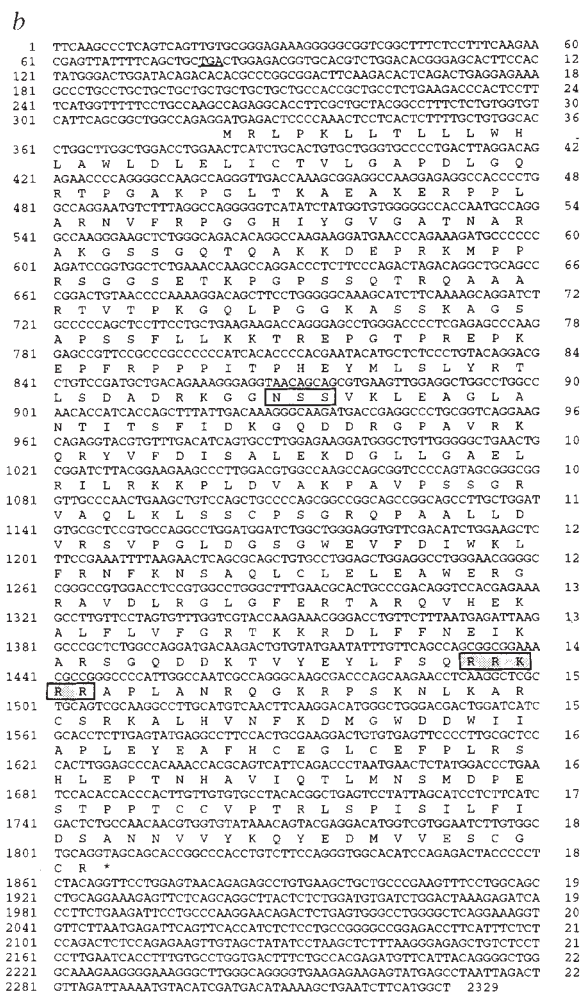


FIG. 1 Sequences of three new members of the TGF- β superfamily. **a**, Partial amino-acid sequences of GDF5, GDF6 and GDF7 are aligned beginning at their predicted protease recognition sites (underlined). Dashes denote gaps introduced to maximize the alignment. Numbers indicate amino-acid positions in GDF5. The seven conserved cysteine residues are indicated (*). Identical amino acids are shaded. **b**, Nucleotide sequence of a full-length *Gdf5* cDNA clone. The predicted GDF5 product is a 495-amino-acid, 54.9K protein with a putative proteolytic processing site (shaded box) preceding a 120-amino-acid, 13.6K mature C-terminal polypeptide. A single N-linked glycosylation site is located in the pro-region (open box). An in-frame termination codon upstream of the putative ATG initiation codon is underlined. The poly(A) tail is not shown.

METHODS. The following oligonucleotide primers were used to screen mouse genomic DNA for new members of the TGF- β superfamily by PCR. Corresponding amino-acid sequences are shown in brackets. SJL136: CCGGAATTCGG(G/A/T/C)TGGGA(G/A/A/C)G(G/A/T/C)TGG(G/A/T/G/A/T/C)(G/A/T) [GWE(R/S)W(V/I/M)(V/I/M)]; SJL121: CCGGAATTC(G/A)-CAICG(C/A)CA(T/C)TC(G/A)TCIACIACCAT(G/A)TC(T/C)TC(G/A)TA [reverse complement: YEDMVVDEC GC]; SJL141: CCGGAATTCGGITGG(G/C/A)-G(A/T/C)(G/A)(A/T/C)TGG(A/G/T)(A/G/T)(T/G)CICC [GW(H/Q/N/K/D/E)(D/N)W(V/I/M)(V/I/M)(A/S/P)]; SJL145: CCGGAATTC(G/A)CAI(G/C)(G/A)CAIG(C/A)(G/A/T/C)TCAI(G/A)(T/C)CAT [reverse complement: M(V/I/M/T/A)(V/D/E)(A/S)(C/G)(A/C)]; and SJL146: CCGGAATTC(G/A)CAI(G/C)-M(V/I/M/T/A)(V/R/S)(A/S)(G/A)(C). PCR was carried out at 94 °C for 1 min, 50 °C for 2 min, and 72 °C for 2 or 3.5 min for 40 cycles with 2 μ g CD-1 mouse genomic DNA as template. Initial GDF5, GDF6 and GDF7 sequences were identified using primers SJL136 and SJL121, SJL141 and SJL145, and SJL141 and SJL146, respectively. The GDF6 and GDF7 sequences were used to screen a mouse BALB/c genomic library from A. Lanahan. GDF5 sequences were used to screen a cDNA library constructed in the λ 2APII vector (Stratagene) from day 12.5 post-coitum (p.c.) CD-1 mouse embryo poly(A)⁺ RNA. PCR products, genomic clones and the longest cDNA clone were sequenced as described²⁰.



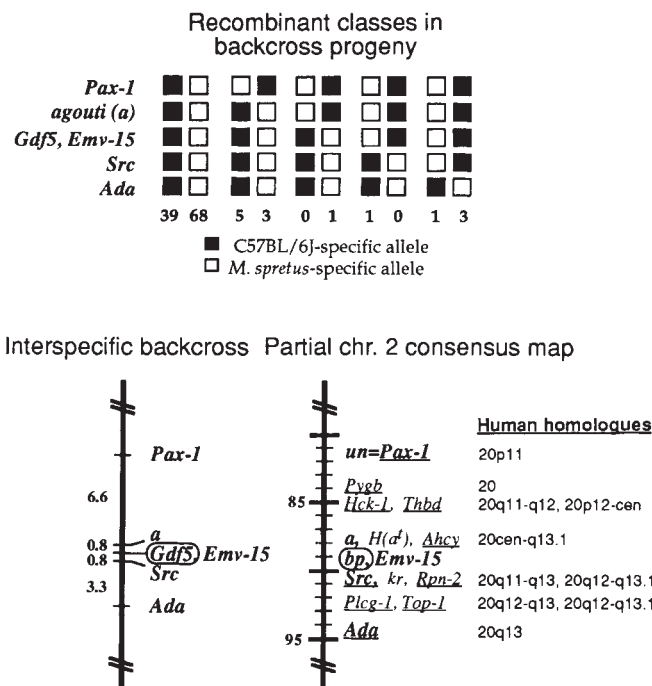


FIG. 2 *Gdf5* maps in the *bp* region of mouse chromosome 2. Top, recombination mapping places *Gdf5* between the *agouti* and *Src* loci on chromosome 2, closely linked to *Emv-15*; bottom, comparison of *Gdf5* map position with a partial consensus linkage map of distal mouse chromosome 2 (ref. 14). The *bp* locus has also been placed between *agouti* and *Src*, closely linked to *Emv-15*. Several underlined loci both proximal and distal to this region have been mapped to chromosome 20 in humans (map locations shown on the right)¹⁴. METHODS. DNA was prepared from progeny of an interspecific backcross between (C57BL/6J × *M. spretus*) F₁ females and C57BL/6J males, digested with *HincII*, and analysed by Southern blot hybridization with a *Gdf5* cDNA probe (nucleotides 1,997–2,329). This probe hybridized to a 3.7-kb *HincII* fragment in C57BL/6J DNA, a 2.6-kb fragment in *M. spretus* DNA, and to one or both fragments in backcross progeny. The inheritance of this restriction-fragment length polymorphism in 121 backcross progeny was compared with the inheritance patterns of over 2,000 other markers previously typed on the same animals²¹, including *Pax-1*, *a*, *Emv-15*, *Src* and *Ada*^{22,23}. Columns of black and white boxes represent chromosome types inherited from the F₁ hybrid parent. Numbers beneath the columns represent the number of backcross progeny observed with each chromosome type.

shorter than controls. The reduction in length of the feet is largely the result of altered patterning of segments in the digits. In place of the proximal and medial phalanges is a single bone that has been described previously as resulting from a fusion of the proximal and medial phalangeal condensations (Fig. 4b)². Additional defects in the length of the metacarpals and metatarsals and slight disorganization of the carpals and tarsals are also present. In contrast, the axial skeleton is largely unaffected by the mutations.

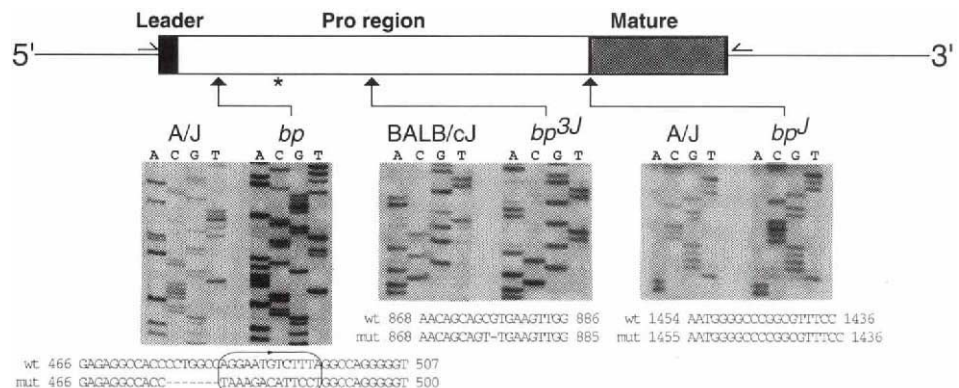
Previous studies have shown that defects in brachypod mice are first detectable around day 12 of gestation², when mesenchyme first aggregates into outlines of future digit elements. Digit condensations in brachypod mice are thin, malformed, and slow to initiate chondrogenesis. In addition,

mesenchyme from brachypod mice shows a reduced ability to form aggregates and cartilaginous nodules *in vitro*^{9,15}. To determine the sites of *Gdf5* expression in wild-type embryos, serial sections of day-12.5 embryos were analysed by *in situ* hybridization. *Gdf5* transcripts were detected in the limbs in distal precartilaginous mesenchymal condensations and in the perichondrium of more proximal skeletal structures (Fig. 4c–f). No other major sites of hybridization were detected in axial skeletal structures or soft tissues.

The sequencing studies reported here provide strong genetic evidence that the skeletal alterations in *bp* mice are the result of mutations in *Gdf5*. This conclusion is further supported by the map location of *Gdf5*, its homology to BMPs, its specific expression in the mesenchymal condensations that give rise to the limb

FIG. 3 Three independent *bp* mutations disrupt *Gdf5* coding sequences. Top, schematic diagram of the *Gdf5* cDNA showing positions of sequence alterations in *bp* alleles. All three mutations result in frameshifts and premature translational termination in the GDF5 open reading frame. The *bp* mutation occurred spontaneously on an outbred mouse strain in 1952¹. The *bp'* and *bp^{3J}* mutations occurred spontaneously in the highly inbred A/J and BALB/cJ wild-type strains in 1975 and 1993, respectively²⁴. Position 613 (asterisk) is polymorphic in different strains, resulting in a serine residue in CD-1, BALB/cJ and *bp^{3J}* mice, and a proline residue in the A/J, *bp'* and *bp* strains. Bottom, details of frameshift mutations. Wild-type and mutant sequence information is presented beneath autoradiograms demonstrating the sequence changes. The reverse complement of bases 1,436–1,454 is shown.

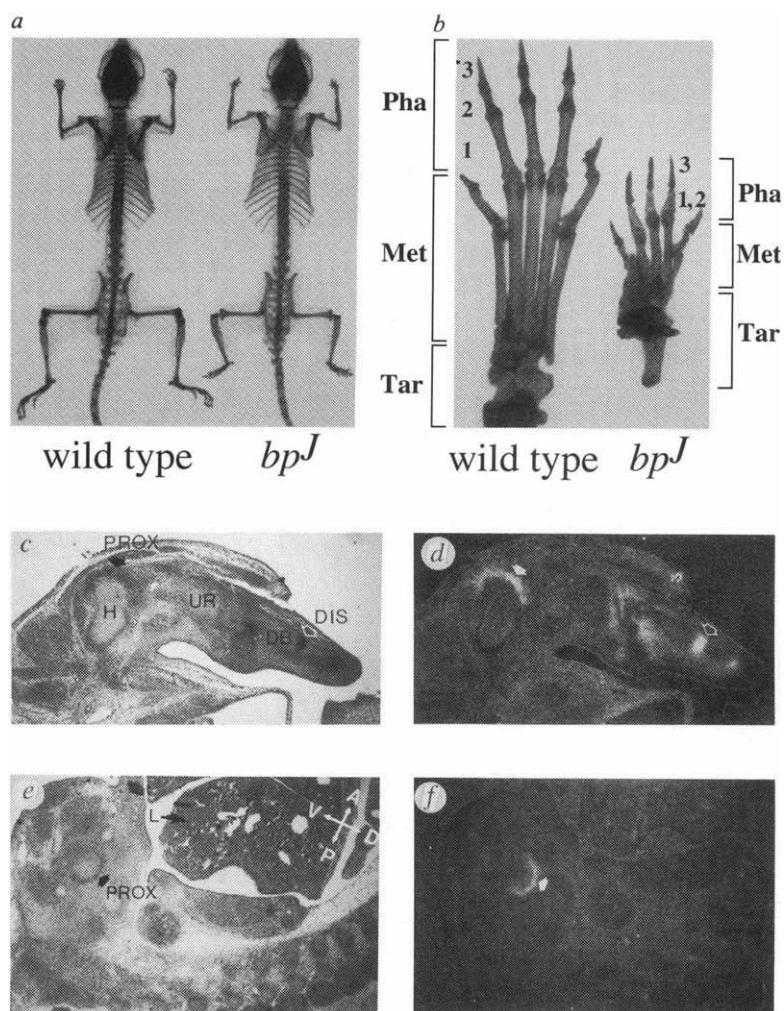
METHODS. *Gdf5* coding sequences were amplified from control and mutant RNA using reverse transcription and PCR. RNA was isolated from brains using RNazol (CINNA-BIOTECX). First-strand reverse transcription reactions used primers located from 1,869 to 1,850 or 1,575 to 1,554 in the wild-type *Gdf5* cDNA sequence. cDNA reactions were then amplified with primer pairs 1,306–1,322 and 1,869–1,850, or 272–289 and



1,575–1,554. Second-round amplifications used primers 1,364–1,384 and 1,869–1,850, or 290–308 and 1,497–1,480. Amplified PCR products were cut from Sea Plaque (FMC) agarose and sequenced directly using the Sequenase Version 2.0 sequencing kit (USB). Sequencing primers correspond to positions 294–311, 767–783, 846–829, 1,364–1,384, 1,497–1,480 and 1,869–1,850 in the cDNA. Arrows bracket the region completely sequenced in two control strains and the three mutant alleles (position 320 to 1,814). Each frameshift mutation was confirmed on both strands and in at least two independent reverse-transcription PCR reactions.

FIG. 4 Skeletal phenotype of *bp*^J mutant and *Gdf5* expression in the developing limb. **a**, Alizarin red skeletal preparations from a representative *bp*^J homozygote and an age-matched wild-type animal from the parental A/J inbred strain. The long bones are reduced and the fibula is shortened proximally. The bones of the axial skeleton are unaffected. **b**, close-up view of right hind feet. The most prominent alteration is a change in the number of the phalanges in *bp*^J homozygotes. In addition, metatarsals are reduced in length and the tarsals are irregular in shape. **c–f**, Expression of *Gdf5* in limb mesenchyme of day-12.5 p.c. mouse embryos. Bright-field (c, e) and dark-field (d, f) photomicrographs of transverse (c, d) and sagittal (e, f) sections, showing views through forelimb and posterior end of embryo, respectively, after hybridization with ³⁵S-labelled *Gdf5* antisense probe. Serial sections revealed hybridization to be localized to proximal (PROX, filled arrows) and distal (DIS, open arrows) mesenchyme in the forelimb (c, d) and hindlimb (e, f). Anterior–posterior (A–P) and dorsal–ventral (D–V) embryonic axes are indicated (e). UR, H and DB indicate primordia of ulna and radius, humerus, and digital bones; L indicates liver. *Gdf5* sense control probes produced no signals on adjacent sections (data not shown).

METHODS. Skeletons of mice were prepared in 1% (w/v) potassium hydroxide and stained using Alizarin red²⁵. Day-12.5 p.c. female mouse embryos were fixed, embedded in paraffin, sectioned and hybridized as described²⁶ to sense or antisense RNA probes (4×10^5 c.p.m. μl^{-1}) transcribed from templates containing nucleotides 308–1,446 of the *Gdf5* cDNA clone. Slides were developed after a 4–6-week exposure time to Kodak NTB3 emulsion and were stained with haematoxylin and eosin.



skeleton, and previous experiments showing that the *bp* mutation acts cell non-autonomously in cultured cells and chimaeric mice^{8,9}. The remarkable ability of BMPs to induce ectopic bone and cartilage⁶ and the defects in skeletal structures caused by *Bmp5* mutations in *short ear* mice⁷ has suggested that BMPs are the normal signals used to initiate bone and cartilage formation during embryonic development¹⁶. Our results strongly support this model and provide evidence that BMP-related genes are also required for skeletal patterning in the vertebrate limb. Strikingly, although the *brachypodism* and *short ear* mutations both disrupt the condensation of mesenchyme cells into outlines of particular skeletal elements, they do so in completely different regions of the embryo: *short ear* null mutations alter the size and shape of ears, sternum, ribs and vertebral processes, but do not alter limb bone lengths or digit morphology, whereas *bp* null mutations alter bone lengths and the number of segments in the digits of all four limbs, but do not affect ear, sternum, rib or vertebral morphology. The skeleton of higher animals thus appears to be a mosaic structure that is built from the composite patterns of activity of different BMP-like proteins. Changes in the activity of particular BMP family members may provide a general mechanism for altering the number and morphology of individual skeletal elements during development and evolution.

Although *Gdf5* transcripts are present in a variety of non-skeletal tissues in adult mice (including uterus, placenta, oviduct, brain, thymus, heart, lung, kidney and adrenal gland; data not shown), brachypod mice are fertile and do not show obvious behavioural abnormalities or striking morphological changes in soft tissues. More detailed studies will be required to determine

whether subtle defects are present, or whether related family members can compensate for *GDF5* deficiencies in soft tissues. A number of human brachydactyly syndromes are known that also disrupt the size or number of phalanges and limb bones without producing obvious abnormalities in other organ systems^{17–19}. Direct analysis of *Gdf5* sequences or linkage studies with chromosome 20 markers (Fig. 2) can now be used to test whether any of these human syndromes are also due to *Gdf5* mutations. □

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- Landauer, W. J. *Hered.* **43**, 293–298 (1952).
- Grüneberg, H. & Lee, A. J. *J. Embryol. exp. Morph.* **30**, 119–141 (1973).
- Hinchliffe, J. R. & Johnson, D. R. *The Development of the Vertebrate Limb* (Clarendon, Oxford, 1980).
- Johnson, D. R. *The Genetics of the Skeleton. Animal Models of Skeletal Development* (Clarendon, Oxford, 1986).
- Massague, J. A. *Rev. Cell Biol.* **6**, 597–641 (1990).
- Rosen, V. & Thies, R. S. *Trends Genet.* **8**, 97–102 (1992).
- Kingsley, D. M. *et al. Cell* **71**, 399–410 (1992).
- Malinina, N. A., Kindiakov, B. N. & Koniukhov, B. V. *Ontogenez* **15**, 514–521 (1984).
- Owens, E. M. & Solursh, M. *Dev. Biol.* **91**, 376–388 (1982).
- Ozkaynak, E. *et al. EMBO J.* **9**, 2085–2093 (1990).
- Celeste, A. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **87**, 9843–9847 (1990).
- Ozkaynak, E. *et al. J. Biol. Chem.* **267**, 25220–25227 (1992).
- Dickinson, M. E. *et al. Genomics* **6**, 505–520 (1990).
- Siracusa, L. D. & Abbott, C. M. *Mamm. Genome* **4**, S31–46 (1993).
- Elmer, W. A. & Selleck, D. K. *J. Embryol. exp. Morph.* **33**, 371–386 (1975).
- Kingsley, D. M. *Trends Genet.* **10**, 16–21 (1994).
- Temtamy, S. A. & McKusick, V. A. *The Genetics of Hand Malformations 1–619* (Liss, New York, 1978).
- Ahmad, M., Abbas, H., Wahab, A. & Haque, S. *Am. J. med. Genet.* **36**, 292–296 (1990).
- Osebold, W. R., Remondini, D. J., Lester, E. L., Spranger, J. W. & Opitz, J. M. *Am. J. med. Genet.* **22**, 791–809 (1985).
- McPherron, A. C. & Lee, S.-J. *J. Biol. Chem.* **268**, 3444–3449 (1993).

21. Copeland, N. G. et al. *Science* **262**, 57–66 (1993).
22. Siracusa, L. D., Buchberg, A. M., Copeland, N. G. & Jenkins, N. A. *Genetics* **122**, 669–679 (1989).
23. Siracusa, L. D. et al. *Genomics* **6**, 491–504 (1990).
24. Lane, P. W., Mobraaten, L. E. & Neleski, L. A. *List of Mutations and Mutant Stocks of the Mouse Maintained at The Jackson Laboratory* (Jackson Laboratory, Bar Harbor, ME, 1993).
25. Green, M. C. *Ohio J. Sci.* **52**, 31–33 (1952).
26. Jones, C. M., Lyons, K. M. & Hogan, B. L. *Development* **111**, 531–542 (1991).

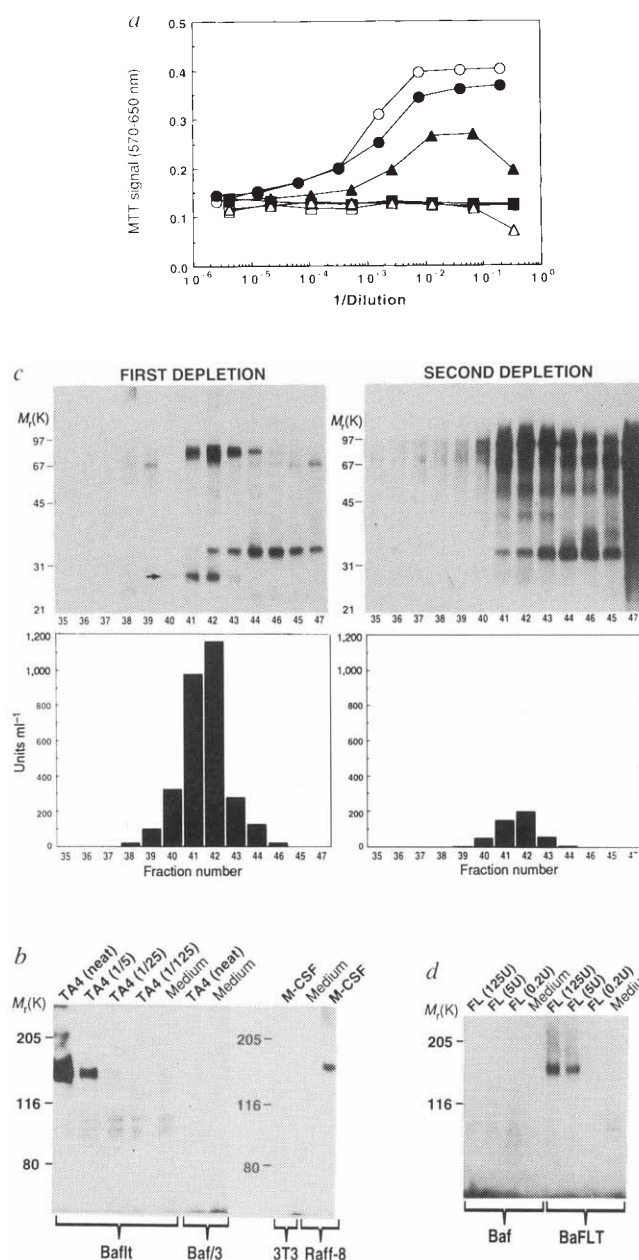
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Ligand for FLT3/FLK2 receptor tyrosine kinase regulates growth of haematopoietic stem cells and is encoded by variant RNAs

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THE FLT3/FLK2 receptor tyrosine kinase is closely related to two receptors, c-Kit and c-Fms, which function with their respective ligands, Kit ligand and macrophage colony-stimulating factor to control differentiation of haematopoietic and non-haematopoietic cells^{1–5}. FLT3/FLK2 is thought to be present on haematopoietic stem cells and found in brain, placenta and testis^{3–5}. We have purified to homogeneity and partially sequenced a soluble form of the FLT3/FLK2 ligand produced by mouse thymic stromal cells. We isolated several mouse and human complementary DNAs that



encode polypeptides with identical N termini and different C termini. Some variants contain hydrophobic transmembrane segments, suggesting that processing may be required to release soluble ligand. The purified ligand enhances the response of mouse stem cells and a primitive human progenitor cell population to other growth factors such as interleukins IL-3 and IL-6 and to granulocyte-macrophage colony-stimulating factor, and also stimulates fetal thymocytes.

To develop an assay for the FLT3/FLK2 ligand (FL) and to identify producer cells, we transfected the pro-B cell line Ba/F3 (ref. 6) with a cDNA clone encoding mouse FLT3/FLK2. Stable transformants, called Bafit, were then used as target cells in a proliferation assay with conditioned medium from several mouse stromal cell lines. The thymic stromal cell line TA4 produced a stimulatory activity for Bafit cells but not the parental Ba/F3 cells (Fig. 1a). TA4-conditioned medium could also induce tyrosine autophosphorylation of the receptor expressed on Bafit cells,

detectable after immunoprecipitation of the receptor (Fig. 1b). These results show that the FL activity in TA4-conditioned medium has the properties expected for the ligand, namely specificity for cells expressing the receptor and the ability to induce receptor autophosphorylation⁷.

FL was affinity-absorbed onto immobilized receptor from concentrated TA4 conditioned medium using two successive receptor columns. Under our conditions >90% of the FL activity was typically removed by the first of the two columns. To aid in the identification of the ligand, the two column eluates were separately chromatographed on reverse-phase HPLC, and the fractions assayed for biological activity and analysed by SDS-PAGE (Fig. 1c). SDS-PAGE analysis of samples from the primary depletion revealed a prominent band at a position corresponding to M_r 30,000 (30K), whose elution pattern coincided with the peak of biological activity. Similar analysis of samples from the secondary depletion showed only a trace of

a

moT110/T118

GAATTCGCGCCGCGTCGAGCCTGGCGGGACTGAGCCCGAGACCTGCCCTCCTGTCACTTCCAAGAACCCTGTCACAGGCATGAGGGGTCCCGGCAGAG		ATGACAGTG METThrVal	3
CTGGCGCCAGCCTGGAGCCCAATTCCTCC	CTGTTGCTGTGTTGCTGCTGCTGAGTCTCT	TGCCTGCGGGGACACCTGACTGTTACTTC	AGCCACAGTCCCCTCTCTCAACTTCAAA
LeuAlaProAlaTrpSerProAsnSerSer	LeuLeuLeuLeuLeuLeuLeuSerPro	CysLeuArgGlyThrProAspCysTyrPhe	SerHisSerProIleSerSerAsnPheLys
43			
GTGAAGTTTATAGAGTTGACTGACCACTG	CTTAAAGATTACCCAGTCACTGTGGCGTC	AATCTTCAGGACGAGAAGCACTGCAAGGCC	TTGTGGAGCCTCTTCTAGCCAGCGCTGG
VallysPheArgGluLeuThrAspHisLeu	LeuLysAspTyrProValThrValAlaVal	AsnLeuGlnAspGluLysHisCysLysAla	LeuTrpSerLeuPheLeuAlaGlnArgTrp
83			
ATAGAGCAACTGAAGACTGTGGCAGGGTCT	AAGATGCAACCGTCTTGGAGACGCTCAAC	ACCGAGATACATTTGTCACTCATGTACC	TTCCAGCCCTTACCAGAATGTCTGCGATTC
IleGluGlnLeuLysThrValAlaGlySer	LysMetGlnThrLeuLeuGluAspValAsn	ThrGluIleHisPheValThrSerCysThr	PheGlnProLeuProGluCysLeuArgPhe
123			
GTCCAGCAACATCTCCACCTCTCTGAAG	GACACCTGCACACAGCTGCTGCTCTGAAG	CCCTGTATCGGGAAGGCTGCCAGAATTC	TCTCGGTGCCTGGAGGTGCAGTGCCAGCCG
ValGlnThrAsnIleSerHisLeuLeuLys	AspThrCysThrGlnLeuLeuAlaLeuLys	ProCysIleGlyLysAlaCysGlnAsnPhe	SerArgCysLeuGluValGlnCysGlnPro
163			
T110			
GACTCTCCACCTGCTGCCCAAGGAGT	CCCATAGCCCTAGAGCCACGGAGCTCCCA	GAGCCTCGGCCAGGCACTGTGTCTCTCT	CTGCTGCTGCTGCTGCTCTCACACTGGTG
AspSerSerThrLeuLeuProProArgSer	ProIleAlaLeuGluAlaThrGluLeuPro	GluProArgProArgGlnLeuLeuLeuLeu	LeuLeuLeuLeuLeuProLeuThrLeuVal
203			
CTGCTGGCAGCCCTGGGCTCTCGCTGG	CAAGGGCAAGAAGGAGGGGGAGCTCCAC	CCTGGGTGCCCTCCCTCCCATCCCTAG	
LeuLeuAlaAlaAlaTrpGlyLeuArgTrp	GlnArgAlaArgArgArgGlyGluLeuHis	ProGlyValProLeuProSerHisPro...	232
T118			
GGTAACGGTGGCCAGAGCCAGCACCAT	GGTGCCACAGGCTCACAGCCACAGCCTTG	CTAACTGTGTGCCAGGCTTCTGCTCCCA	CTAGTTGGCACTTCACACATGTTCTTCTC
GlyAsnGlyGlyProArgAlaGlnHisHis	GlyAlaThrArgLeuThrAlaThrAlaLeu	LeuThrValCysProGlyLeuLeuLeuPro	LeuValGlyThrSerHisMetPhePheLeu
203			
CCTTATTTCTCTCTTTCTTTCTTTT	TAAAGATGTATCTTTATGTGTGA		
ProTyrPheLeuSerPheLeuSerSerPhe	LeuLysMetTyrLeuTyrVal...		220

b

huS86/S109

SP		GAAAGGGCTGTACCCGGCTTGGCCCTTCCACCCCACTGGGGCAAGCCTGACCCGGCGACAGGAGGCATGAGGGGCCCCGGCCGAA	
ATGACAGTGCTGGCGCCAGCCTGGAGCCCA	ACAACCTATCTCCTCTGCTGCTGCTGCTG	AGCTCGGGACTCAGTGGGACCCAGGACTGC	TCCTTCCAACACAGCCCCATCTCTCCGAC
METThrValLeuAlaProAlaTrpSerPro	ThrThrTyrLeuLeuLeuLeuLeuLeuLeu	SerSerGlyLeuMetGlyThrGlnAspCys	SerPheGlnHisSerProIleSerSerAsp
40			
TTGCTGTCAAATCCGTGAGCTGTCTGAC	TACCTGCTTCAAGATTACCCAGTCACCGTG	GCCTCCAACCTGCAGGACGAGGAGCTCTGC	GGGGCGCTCTGGCGGCTGGTCTGGCACAG
PheAlaValLysIleArgGluLeuSerAsp	TyrLeuLeuGlnAspTyrProValThrVal	AlaSerAsnLeuGlnAspGluLeuLeuCys	GlyAlaLeuTrpArgLeuValLeuAlaGln
80			
CGCTGGATGGAGCGGCTCAAGACTGTGCT	GGGTCCAAGATGCAAGGCTTGTGGAGCGC	GTGAACACGGAGATACACTTTGTACCAAA	TGTGCTTTTTCAGCCCCCCCCAGCTGTCTT
ArgTrpMetGluArgLeuLysThrValAla	GlySerLysMetGlnGlyLeuLeuGluArg	ValAsnThrGluIleHisPheValThrLys	CysAlaPheGlnProProProSerCysLeu
120			
CGTTCTGTCAGACCAATCTCCCGCTC	CTGCAGGAGACCTCCGAGCAGCTGGTGGC	CTGAAGCCCTGGATCACTCGCCAGAATTC	TCCCGGTGCCTGGAGCTGCAGTGTACGCC
ArgPheValGlnThrAsnIleSerArgLeu	LeuGlnGluThrSerGluGlnLeuValAla	LeuLysProTrpIleThrArgGlnAsnPhe	SerArgCysLeuGluLeuGlnCysGlnPro
160			
S86			
GACTCTCAACCTGCCACCCCATGGAGT	CCCCGGCCCTGGAGGCCACAGCCCGACA	GCCCGCAGCCCCCTGCTCTCTCTACTG	CTGCTGCCCGTGGGCTCCTGCTGCTGGC
AspSerSerThrLeuProProProTrpSer	ProArgProLeuGluAlaThrAlaProThr	AlaProGlnProProLeuLeuLeuLeuLeu	LeuLeuProValGlyLeuLeuLeuAla
200			
GCTGCTGTGCTGCTGCTGCTGCTGCTG	CGGCGGAGGACACCCCGCTGGGAGCAG	GTGCCCCCGTCCCCAGTCCCCAGGACCTG	CTGCTTGTGGAGCACTGA
AlaAlaTrpCysLeuHisTrpGlnArgThr	ArgArgArgThrProArgProGlyGluGln	ValProProValProSerProGlnAspLeu	LeuLeuValGluHis...
235			
S109			
GGTGGCCCCCTCCAGTCCCGAGGACCT	GCTGCTTGTGGAGCACTGACCTGGCCAAAG	CCTCATCTGGGGAGGATAGTGGGCACAC	AGAGGGAGTCCAGCCAGGAGGATGCATA
GlyAlaProArgProGlnSerProGlyPro	AlaAlaCysGlyAlaLeuThrTrpProArg	ProHisProGlyGluAspThrGluAlaHis	ArgGlyGluSerProAlaArgGlyCysIle
200			
GCCTGGACACAGAGGAAGTGGCTAGAGC	CGGTCCCTTCTTGGGCCCTCTCATTCC	TCCCCAGAATGGAGGCAACGCCAATCCA	GCACCGGCCCATTTACCAACTCTGTACA
AlaTrpThrGlnArgLysLeuAlaArgGly	ArgSerLeuProTrpAlaProLeuIlePro	SerProGluTrpArgGlnArgGlnAsnPro	AlaProAlaProPheThrGlnLeuCysThr
240			
AAGCCCTTGTCCCATGA			
LysProLeuSerPro...	245		

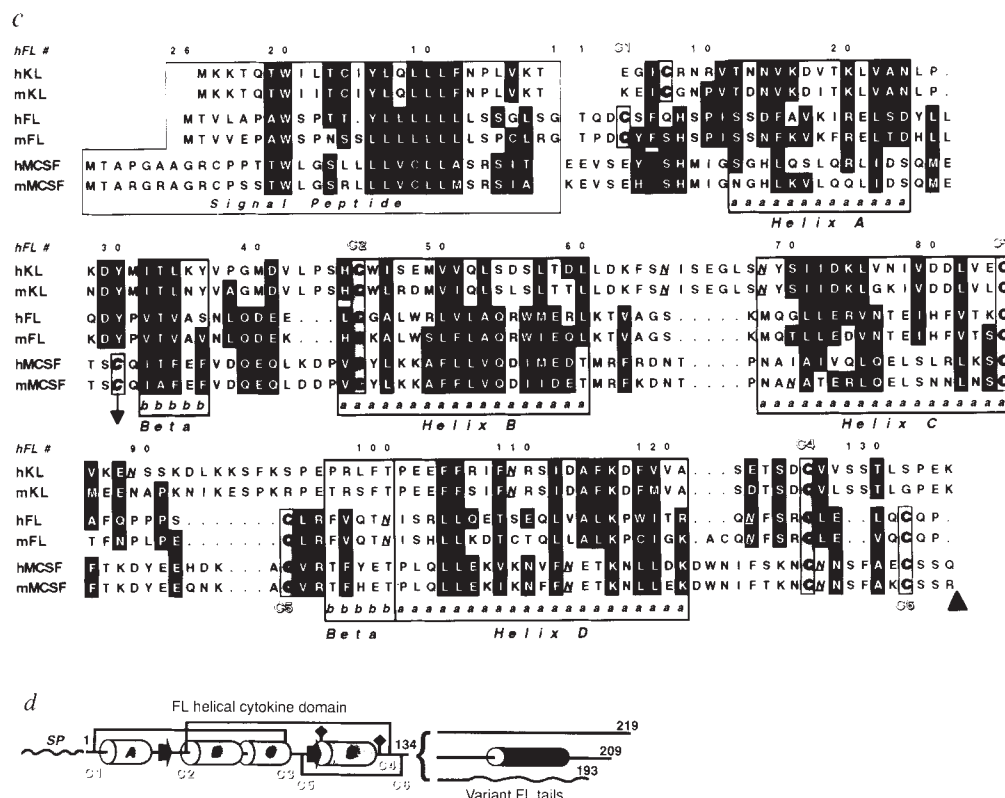
this species, with minimal activity. The native ligand is a non-disulphide-linked 65K homodimer (data not shown). Under our conditions, FL purified >300,000-fold has a specific activity of 3.0×10^6 units mg^{-1} and can induce tyrosine autophosphorylation of FLT3/FLK2 receptor on Baft cells (Fig. 1d). Forty-two N-terminal residues of FL isolated by preparative SDS-PAGE were deduced from combined direct sequencing and overlapping of internal peptide sequences; in addition two non-contiguous peptide sequences were determined (Fig. 2a).

Degenerate oligonucleotide primers were designed based on the N-terminal 42 residues and used to amplify by polymerase chain reaction (PCR) an FL cDNA of sequence corresponding to the peptide sequence. A second, longer PCR product was isolated using primers derived from a non-contiguous downstream peptide. These 5' cDNAs were used as probes to isolate two clones, T118 and T110, from TA4 cDNA libraries by hybridization screening of ~500,000 clones. The first 163 codons

of the T118 and T110 open reading frames are identical but the sequences then diverge, presumably because of alternative splicing, to encode two distinct C termini (Fig. 2a). The 232-residue T110 polypeptide is the longer and contains a putative transmembrane segment of 22 hydrophobic amino acids followed by a cytoplasmic tail. The C terminus of the 220-residue T118 polypeptide is hydrophobic, so it may also be membrane-associated.

Human FL cDNAs were isolated by hybridization with mouse FL cDNA probes using a cDNA library from a human thymic stromal cell line (SV48)⁸ with FL activity. Two clones, S86 and S109 had identical open reading frames for the first 160 codons apart from a 28-base-pair (bp) insertion in S109; the sequences then diverge at the same position as the mouse clones to encode different C termini (Fig. 2b). S86 encodes a polypeptide of 235 amino acids which is homologous to mouse clone T110 (66% identical) and includes a similar transmembrane segment. S109

FIG. 2 Structure of FL. a, Nucleotide and deduced protein sequences of mouse cDNAs T110 and T118. Peptide sequences that were obtained from purified FL are underlined. Boxed sequences indicate the signal peptide (SP) and transmembrane (TM) region. Bold lettering indicates the point at which the sequences diverge. b, Nucleotide and deduced protein sequences of human cDNAs S86 and S109. Clone S109 contains a 28-bp insertion (asterisk) containing a stop codon; sequence not shown. c, Comparison of predicted amino-acid sequences of human (h) and mouse (m) FL with the equivalent signal peptide and cytokine domain chains of KL and M-CSF⁹. Based on an earlier structural alignment⁹ and the X-ray structure of hM-CSF¹⁰, regions of FL predicted to form the α -helical cytokine fold¹¹ are labelled A-D; two short stretches of β -strand are also marked. Four conserved cysteines in grey boxes are marked above the alignment by outline letters C1-C4; two additional cysteines present only in FL and M-CSF are named below as C5 and C6. An italicized cysteine in M-CSF above the arrow covalently links the M-CSF dimers^{9,11}; this residue is missing in KL and FL. Similar residues in the alignment are denoted by reverse lettering. Potential N-glycosylation sites are indicated by italicized underlined asparagines. A black triangle at the end of the alignment marks the point of divergence of variant forms in KL, M-CSF⁹ and FL. d, Diagrammatic representation of the predicted structure and chain variants of FL. The α -helices are drawn as cylinders and labelled as in c; short β -strands are drawn as ribbon arrows. The predicted KL/M-CSF-like disulphide bridge in the FL helical cytokine domain (C1-C3, C2-C4 and C5-C6)⁹ are marked above and below the chain. Diamonds indicate the location of potential N-glycosylation sites in FL. SP denotes the signal peptide, TM marks the hydrophobic transmembrane segment and a wavy C terminus symbolizes the hydrophobic tail of mouse clone T118.



METHODS. 5' FL cDNAs were isolated by PCR using plasmid DNA from a TA4 cDNA library as a template with a 5' vector-derived primer and a 3' degenerate primer encoding the peptide NLQDEK. 10% of this product was reamplified using a 5' degenerate primer encoding the

peptide TPDCYF and a 3' degenerate primer encoding the peptide YPVTV. A 105-bp PCR product was identified using a radiolabelled internal degenerate oligonucleotide encoding the peptide, NFKVKF. The sequence of this PCR product encodes the expected 35-amino-acid peptide. A 303-bp fragment was generated using PCR with a primer corresponding to the 5' end of the 105-bp fragment and a 3' degenerate primer encoding the peptide FVQTNI. This fragment was used to screen a TA4 cDNA library in λ zap II (Stratagene). T118 and T110, two positive clones containing inserts of ~1.6 and ~1.4 kb respectively, were isolated. Human FL cDNAs were obtained using an 800-bp fragment derived from the coding region of clone T118 to screen 5×10^5 colonies of a human SV48 stromal cell cDNA library under low-stringency conditions¹⁵. Two positive colonies, S86 and S109, containing inserts of ~1.1 and ~0.9 kb respectively, were isolated. All cDNA inserts were subcloned into M13 phage for sequencing of both strands¹⁶. Nucleic acid sequences have been deposited in GenBank with the accession numbers U04806 and U04807.

has an in-frame stop codon in the 28-bp insert, which probably represents an exon resulting from aberrant splicing. Excluding this putative exon, clone S109 encodes a 245-amino-acid polypeptide containing a distinct, predominantly hydrophilic C terminus which probably corresponds to a soluble form of the ligand.

The various mouse and human cDNAs define a family of FL molecules sharing a common N terminus that includes ~135 amino-acid residues (including the signal peptide). Comparative sequencing indicates that the common portion of the FL variants is structurally related to Kit ligand (KL) and macrophage colony-stimulating factor (M-CSF), which adopt the α -helical cytokine fold⁹⁻¹¹, and FL may share the disulphide bridge net-

work of KL and M-CSF^{9,10} (Fig. 2c, d). The divergence point of the mouse and human FL cDNAs occurs at a position, analogous to the variable chain-length region of KL and M-CSF, that may tether the receptor-binding cytokine domains of these molecules to the membrane⁹.

Northern analyses of mouse and human FL messenger RNAs in adult and fetal tissues showed that expression was widespread, being highest in spleen and lung (Fig. 3a-c). The principal mouse and human transcripts were ~1.5 kilobases (kb), with two others of 3.5 and 5.0 kb from mouse. FL mRNAs were found in mouse and human stromal lines and were highest in T-cell lines and peripheral blood mononuclear cells (Fig. 3d, e). In one T-cell

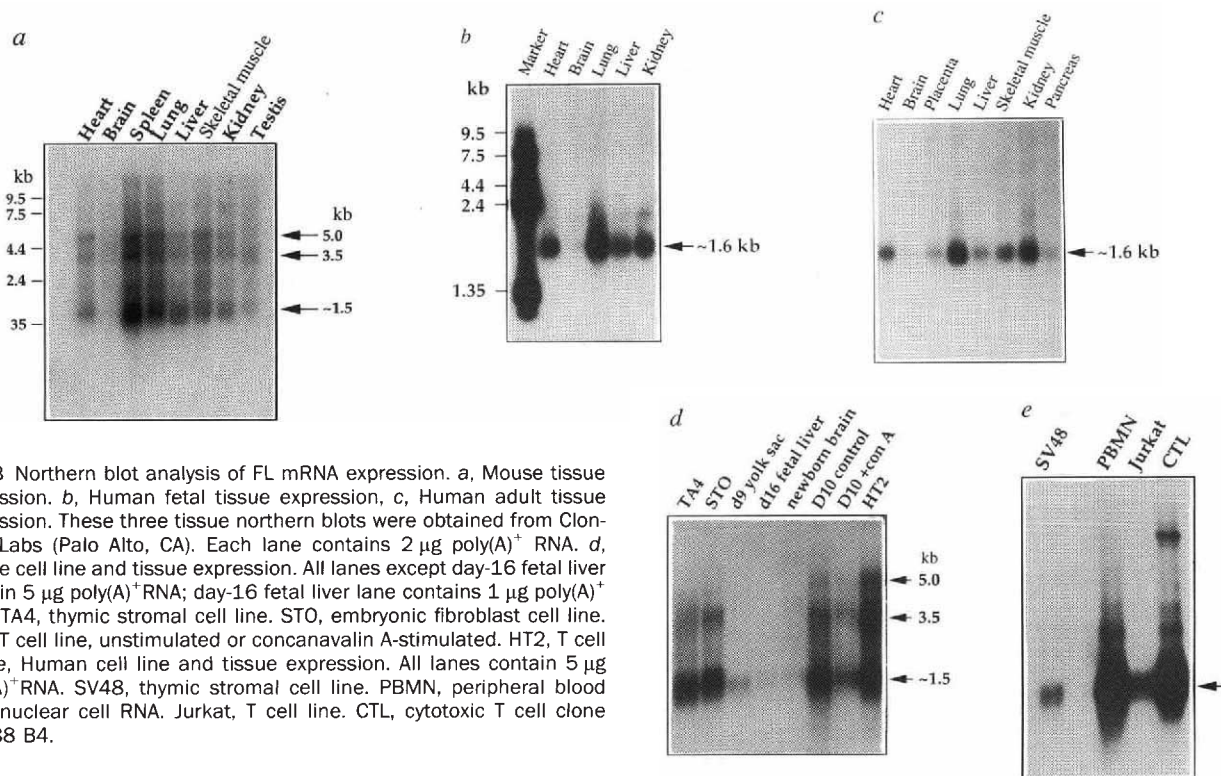


FIG. 3 Northern blot analysis of FL mRNA expression. a, Mouse tissue expression. b, Human fetal tissue expression. c, Human adult tissue expression. These three tissue northern blots were obtained from Clontech Labs (Palo Alto, CA). Each lane contains 2 μ g poly(A)⁺ RNA. d, Mouse cell line and tissue expression. All lanes except day-16 fetal liver contain 5 μ g poly(A)⁺ RNA; day-16 fetal liver lane contains 1 μ g poly(A)⁺ RNA. TA4, thymic stromal cell line. STO, embryonic fibroblast cell line. D10, T cell line, unstimulated or concanavalin A-stimulated. HT2, T cell line. e, Human cell line and tissue expression. All lanes contain 5 μ g poly(A)⁺ RNA. SV48, thymic stromal cell line. PBMN, peripheral blood mononuclear cell RNA. Jurkat, T cell line. CTL, cytotoxic T cell clone A2.288 B4.

FIG. 4 Biological activities of native and recombinant FL. Mouse Baftt (a), or human Baftt (b) stimulation by supernatants from COS cells transfected with mT110S (—▲—), mT110 (—○—), mT118 (—●—), hS86 (—■—), hS109 (—□—), or pME18S vector alone (—△—). c, Autophosphorylation assay using crude and purified natural FL and recombinant soluble FL produced in COP5 fibroblasts of *E. coli*. d, Competitive displacement of ¹²⁵I-mFL binding to Baftt (○) or Ba/F3 (□) cells. 3 × 10⁶ cells and 10⁻¹⁰ M ¹²⁵I-mFL (64 μ Ci per μ g) were used at each point. Filled and unfilled squares are data from two independent experiments and error bars are standard deviations. 100% maximal binding to Baftt cells was 1895 c.p.m. and nonspecific binding was 633 c.p.m. The estimated affinity was $K_d = 7.3 \times 10^{-10}$ M with ~600 sites per cell. e, FL increases colony formation by Thy^oSca-1⁺Lin⁻ mouse bone marrow cells in combination with IL-3 or IL-6. f, FL augments large (HPP-CFC) and small (LPP-CFC) colony formation by CD34⁺CD33⁺Lin⁻ human fetal liver cells. g, FL does not affect the growth of glycophorin⁻ human fetal liver BFU-E. h, FL increases the proliferation of fetal thymocytes in combination with IL-7.

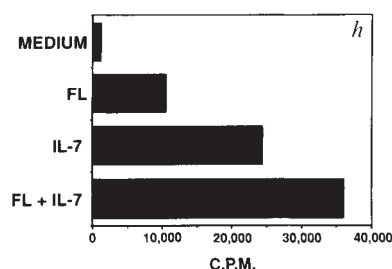
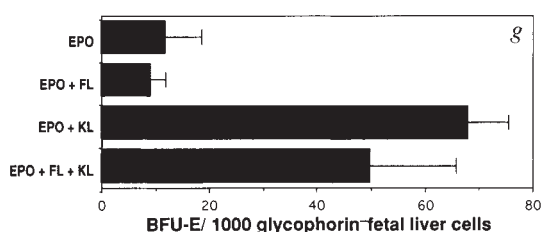
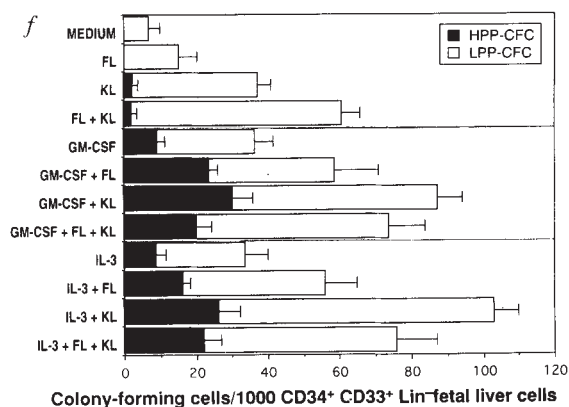
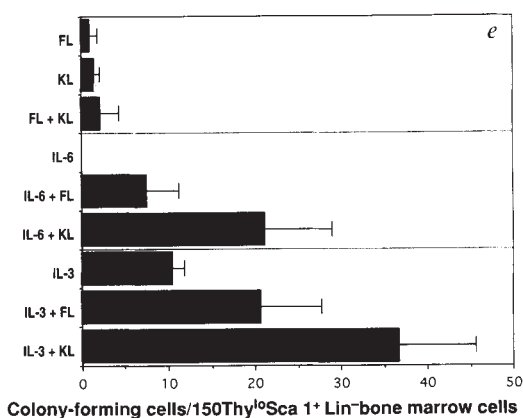
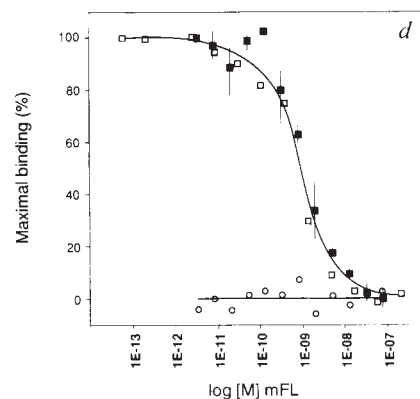
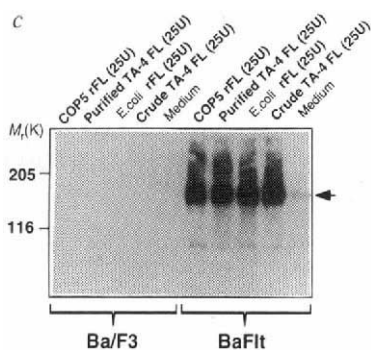
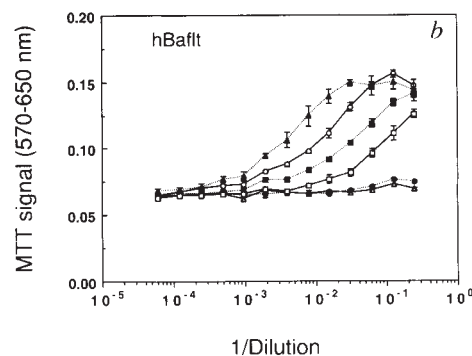
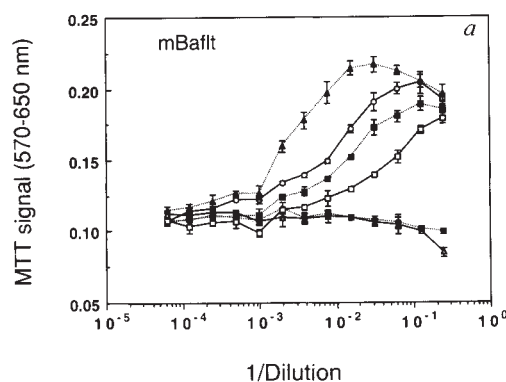
METHODS. cDNA inserts from phage clones T110 and T118 were subcloned into pME18S¹⁵; clones S86 and S109 were isolated in the same vector. Supernatants from the transiently expressing cells were collected 72 h after transfection and assayed on Baftt cells. A soluble form of mouse FL was produced in *E. coli* from clone T118 (residues 28–189). This material was refolded from inclusion bodies, purified by reversed phase chromatography (R. K. and S. M., unpublished), and had a specific activity of 2 × 10⁶ U. per mg in the MTT survival assay.

Procedures for ¹²⁵I-radiolabelling of *E. coli*-derived mFL as well as the receptor binding assay have been described previously^{17,18}. Procedures for sorting Thy^oSca-1⁺Lin⁻ cells¹⁹ and colony-forming assays²⁰ have been described. Growth factors were used at concentrations previously stated²⁰ except for recombinant mKL (R&D Labs, 20 ng ml⁻¹) and purified native mFL (25 U ml⁻¹). Cultures were scored at day 7. Light density human fetal liver cells were depleted of glycophorin⁺ cells as described²¹. Progenitors were sorted from glycophorin⁻ fetal liver cells based on the lack of staining with PE-labelled CD3, C14, CD16, CD20, and CD56 (Lin⁻) (Becton Dickinson) and positive staining with FITC-labelled CD34 (HPCA-2; Becton Dickinson) and TC-labelled CD33 (Caltag Labs). CD34⁺CD33⁺Lin⁻ cells were cultured in the presence of combinations of recombinant hGM-CSF (Schering-Plough; 10 ng ml⁻¹), recombinant hIL-3 (R&D Labs; 20 ng ml⁻¹), native mFL (25 U ml⁻¹) and recombinant hKL (R&D Labs; 20 ng ml⁻¹) for 21 d in agarose cultures²¹. Cultures were scored for LPP-CFC (colonies > 50 cells) and HPP-CFC (colonies > 0.5 mm in diameter). Glycophorin⁻ human fetal liver cells were cultured in the presence of combinations of recombinant hEPO (R&D Labs; 1 U ml⁻¹), purified native mFL and recombinant hKL for 14 d in 1.2% methylcellulose²². All colony data are presented as the mean ± s.d. scored from 3 or 4 cultures. Significant differences were calculated using a paired Student's *t*-test. BALB/c day-14 fetal thymocytes were cultured for 27 h with appropriate recombinant cytokines. mIL-7 was used at 200 U ml⁻¹ and recombinant (*E. coli*) mFL at 100 U ml⁻¹. Cell proliferation was measured by the incorporation of ³H-thymidine and data represents the average of duplicate samples.

line these levels were not inducible with concanavalin A and we could not recover soluble active protein from T-cell conditioned medium.

Mouse clone T110 and its human counterpart S86, when transiently transfected into COS cells, produce soluble activities that induce proliferation of BaFt cells but not Ba/F3 cells (Fig. 4a). When the chain-terminating exon in S109 is removed, it too can direct the synthesis of soluble ligand in COS cells. To confirm

that the portion of the molecule that is similar to KL, M-CSF and other cytokines is functional, we engineered an artificial form of clone T110 without the transmembrane region and cytoplasmic tail. When this clone, T110S, was transfected into COS cells, it gave high soluble FL activity. Mouse clone T118, whose protein has a very hydrophobic tail, gave the lowest expression of soluble activity in COS cells, detectable only when the conditioned medium was concentrated (data not shown). All the



recombinant forms, mouse and human, have similar relative activities when assayed on Ba/F3 cells expressing either the mouse or human receptor (Fig. 4a, b). Like the crude and purified native ligand, recombinant ligand produced by either transfected mammalian fibroblasts (COP5) or *Escherichia coli* can induce tyrosine autophosphorylation of FLT3/FLK2 receptor on Baft cells (Fig. 4c). The K_d of soluble FL for FLT3/FLK2 is 7.3×10^{-10} M, measured by competitive binding with recombinant bacterial FL and Baft cells (Fig. 4d).

We performed clonal assays in methylcellulose using mouse Thy^{1.0}Sca-1⁺ Lin⁻ stem cells purified from bone marrow. There was no induction of colony growth when purified native mouse FL was added alone to this cell population (Fig. 4e), but in combination with the multilineage growth factor IL-3, colony numbers were double those in the presence of IL-3 alone. These conditions produced multilineage colonies, but they did not contain the abundant erythroid cells found with KL and IL-3 (data not shown). There is similar increase in colony numbers when FL is added to IL-6.

When CD34⁺ CD33⁺ Lin⁻ human fetal liver progenitor cells were used, purified native FL alone had little or no stimulatory activity. But, as with mouse stem cells, it had a synergistic effect in combination with IL-3 as well as with granulocyte-macrophage colony-stimulating factor (GM-CSF) (Fig. 4f). In this case, the costimulatory effects of FL were observed on low-proliferative-potential colony-forming cells (LPP-CFC) and on the more primitive high-proliferative-potential colony-forming cells (HPP-CFC). Consistent with the results from mouse stem cells, FL was unable to augment the growth of human fetal liver burst-forming units erythroid (BFU-E) in the presence of erythropoietin (Fig. 4g). These results indicate that FL enhances the response of stem and primitive progenitor cells to other growth factors to generate all myeloid lineages except erythroid cells.

Because FLT3/FLK2 is present on immature lymphoid cells and myeloid cells, we tested the response of thymocytes to purified FL. Mouse adult T-cell precursors have been shown to proliferate in response to IL-7 and KL¹². IL-7 in combination with FL induces significant proliferation in day-14 mouse fetal thymocytes (Fig. 4h) consisting mainly of T-cell precursors, suggesting that FL may have a role in T-cell development.

We have identified the FLT3/FLK2 ligand to help clarify the biological processes controlled by this receptor-ligand pair. Our results indicate that the ligand controls the growth response of haematopoietic progenitor and stem cell populations. Recombinant FL will facilitate studies of the effects of this new cytokine on these and other cells. □

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- Sherr, C. J. *Blood* **75**, 1–12 (1990).
- Witte, O. N. *Cell* **63**, 5–6 (1990).
- Rosnet, O., Marchetto, S., deLapeyriere, O. & Birnbaum, D. *Oncogene* **6**, 1641–1650 (1991).
- Rosnet, O. et al. *Blood* **82**, 1110–1119 (1993).
- Mathews, W., Jordan, C. T., Wiegand, G. W., Pardoll, D. & Lemischka, I. R. *Cell* **65**, 1–20 (1991).
- Palacios, R. & Steinmetz, M. *Cell* **41**, 727–734 (1985).
- Schlessinger, J. & Ullrich, A. *Neuron* **9**, 383–391 (1991).
- Galy, A. H. M., de Waal Malefyt, R., Barcena, A., Mohan-Peterson, S. & Spits, H. *Thymus* **22**, 13–33 (1993).
- Bazan, J. F. *Cell* **65**, 9–10 (1991).
- Pandit, J., Bohm, A., Jancarik, J., Halenbeck, R., Kothe, K. & Kim, S. H. *Science* **258**, 1358–1362 (1992).
- Sprang, S. R. & Bazan, J. F. *Curr. Opin. Struct. Biol.* **3**, 815–827 (1993).
- Godfrey, D. I., Zlotnik, A. & Suda, T. *J. Immunol.* **149**, 2281–2285 (1992).
- Mosmann, T. R. *J. Immunol. Meth.* **65**, 55–63 (1983).
- Maroc, N. et al. *Oncogene* **8**, 909–918 (1991).
- McKenzie, A. N. J. et al. *Proc. Natn. Acad. Sci. U.S.A.* **90**, 3735–3739 (1993).
- Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. Natn. Acad. Sci. U.S.A.* **74**, 5463–5467 (1977).
- Zurawski, S. M. & Zurawski, G. *EMBO J.* **11**, 3905–3910 (1992).
- Zurawski, S. M., Vega, F., Huyghe, B. & Zurawski, G. *EMBO J.* **12**, 2663–2670 (1993).
- Spangrude, G. J., Heimfeld, S. & Weissman, I. L. *Science* **241**, 58–62 (1988).
- Heimfeld, S., Hudak, S., Weissman, I. L. & Rennick, D. *Proc. Natn. Acad. Sci. U.S.A.* **88**, 9902–9906 (1991).
- Barcena, A. et al. *Blood* (in the press).

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Specificity of the thrombin receptor for agonist peptide is defined by its extracellular surface

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G-PROTEIN-COUPLED receptors for catecholamines and some other small ligands are activated when agonists bind to the transmembrane region of the receptor¹. The docking interactions through which peptide agonists activate their receptors are less well characterized^{2–7}. The thrombin receptor is a specialized peptide receptor. It is activated by binding its tethered ligand domain, which is unmasked upon receptor cleavage by thrombin^{8,9}. Human and *Xenopus* thrombin receptor homologues are each selectively activated by the agonist peptide representing their respective tethered ligand domains. Here we identify receptor domains that confer this agonist specificity by replacing the *Xenopus* receptor's amino-terminal exodomain and three extracellular loops with the corresponding human structures. This switches receptor specificity from *Xenopus* to human. The specificity of these thrombin receptors for their respective peptide agonists is thus determined by their extracellular surfaces. Our results indicate that agonist interaction with extracellular domains is important for thrombin receptor activation.

Identification of the interactions by which the thrombin receptor's tethered ligand domain triggers transmembrane signalling is central to understanding signalling by this and perhaps other peptide receptors and may aid the development of new pharmaceuticals for inhibiting thrombosis and perhaps inflammatory and proliferative responses to vascular injury^{10,11}. To begin our structure-activity studies, we isolated a complementary DNA encoding the *Xenopus laevis* homologue of the human thrombin receptor. The tethered ligand domains of the human and *Xenopus* thrombin receptor were strikingly different: SFLRN for human and TFRIFD for *Xenopus* (Fig. 1). As expected, synthetic peptides mimicking these domains were full agonists for activation of their respective receptors^{8,9}. However, each agonist peptide showed surprising selectivity for the receptor of its own species. The human agonist peptide was ~1,500-fold more potent at the human receptor than at the *Xenopus* receptor, and the *Xenopus* peptide was some 30-fold more potent at the *Xenopus* than at the human receptor (Figs 2, 3 and Table 1). These observations indicated that the receptor domains that define agonist specificity might be identified using human-*Xenopus* chimaeric receptors. Because the human agonist peptide functioned poorly at the *Xenopus* receptor, we began by building human sequences into the *Xenopus* receptor and looking for gain of responsiveness to the human agonist peptide.

Replacement of the *Xenopus* thrombin receptor's extracellular face with that of human (Figs 2 and 3) caused a remarkable switch in receptor specificity—a clear-cut reversal in agonist selectivity, with a nearly 3-log gain of potency for the human agonist and 3-log loss of potency for the *Xenopus* agonist. Substitution of individual human receptor domains showed that the amino-terminal exodomain (HAT93 chimaera) and second extracellular loop (HECL2 chimaera) each contributed to this change in specificity, and a chimaera that included both substitu-

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TABLE 1 Determinants of agonist peptide function and specificity

EC ₅₀ (μM) at:	Human receptor (HWT)	Xenopus receptor (HAT75)			
Peptide					
TFRIFD (XWT)	ND	3	SFLIRN	30	100
AFRIFD	ND	25	SFLLFN	10	100
TARIFD	ND	> 300	SFRLFN	10	100
TFAIFD	ND	ND*			
TFRADF	ND	100	TFRIFD (XWT)	90	3
TFRIAD	ND	300	SFRIFD	90	3
TFRIFA	ND	5	TFLIFD	ND*	ND*
			TFRLFD	20	10
SFLLRN (HWT)	3	> 300	TFRIAD	ND*	ND*
TFLLRN	3	> 300	TFLIRN	20	100
SFRLRN	ND*	ND*			

Alanine scanning and chimaeric substitutions reveal a role for agonist peptide positions 4 and 5. Data shown are half-maximal effective concentrations (EC₅₀s) for peptide-stimulated phosphoinositide hydrolysis in stably transfected mammalian cell lines expressing human or *Xenopus* thrombin receptors. To confirm that the specificities of the human and *Xenopus* thrombin receptors held across different expression systems, and because a number of different peptides were to be tested against the same receptors, stable receptor-transfected Rat 1 cell lines were generated as described¹⁴. The human thrombin receptor cDNA used for these experiments was HWT' (Fig. 3). Attempts to generate cell lines that stably expressed the XWT' construct or wild-type *Xenopus* receptor on their surface were unsuccessful, but HAT75 expressed well. Because the agonist specificity of HAT75 was nearly identical to that of the wild-type *Xenopus* receptor in the oocyte system (Fig. 3), we used HAT75 as a surrogate for the wild-type *Xenopus* receptor in the mammalian expression studies. Stable cell lines with comparable surface expression of HWT' and HAT75, as determined by antibody-binding studies¹⁴, were selected. Phosphoinositide turnover was determined as described¹⁴. Incubations with agonist were for 20 min at 37 °C. EC₅₀s for the indicated peptides were estimated from concentration-response curves in which each peptide was added at concentrations ranging from 1 to 300 μM. All points were determined in triplicate and each experiment was replicated twice. ND, not determined.

*Peptide insoluble.

tions (HAT93 plus HECL2) had agonist peptide specificity remarkably similar to that of the human receptor (Fig. 3). In contrast to the HAT93 substitution, HAT75 did not significantly change receptor specificity. Moreover, substitution of human residues 76–93 alone resulted in a phenotype indistinguishable from that of HAT93 (Fig. 3, and data not shown). Taken together, these data show that amino-terminal exodomain residues 76–93 and extracellular loop 2 residues 244–268 are important determinants of thrombin receptor agonist specificity.

We next examined the features of the agonist peptides themselves that are critical for agonist function and receptor specificity. Previous studies with the human agonist peptide

(SFLLRN) showed that the phenyl side chain of F2 and the protonated amino group of S1 were critical for agonist function and that the side chains of L4 and R5 were also important^{12,13}. Alanine scanning of the *Xenopus* peptide revealed a similar structure-activity profile; F2 was crucial and residues 4 and 5, in this case isoleucine and phenylalanine, were also important for agonist function (Table 1). Substitution of a human residue at position 4 in the *Xenopus* peptide yielded gain of potency at the human receptor and loss at the *Xenopus* receptor. Conversely, substitution of *Xenopus* residues at positions 4 or 5 in the human peptide yielded loss of function at the human receptor and gain at *Xenopus*. These and other substitutions show that residues 4

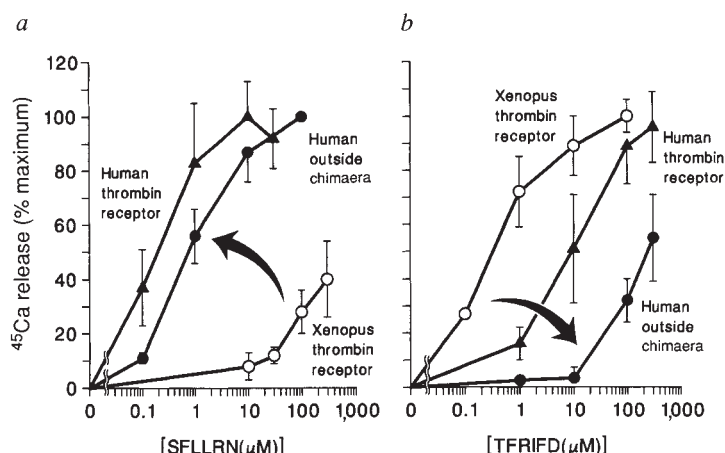
FIG. 1 Comparison of the human (H) and *Xenopus* (X) thrombin receptors' predicted amino-acid sequences.

Domains predicted to be extracellular are overlined. Spaces were introduced to improve alignment or to denote different domains; (/) indicates the thrombin cleavage sites that mediate receptor activation (see below); (-) represents human residues identical to the *Xenopus* sequence (overall amino-acid identity, 53%). This alignment revealed that the *Xenopus* receptor contained an unusual thrombin cleavage site with a lysine at the P1 position, an agonist peptide domain that was remarkably different apart from the critical phenylalanine at position 2, and a hirudin-like domain identical to the sequence in hirudin's carboxyl tail and known to bind thrombin's fibrinogen exosite¹⁵. Identification of the thrombin cleavage site (/) and agonist peptide domain was confirmed by demonstrating that oocytes expressing *Xenopus* thrombin receptors rendered uncleavable with K42A or T43P substitutions could not be activated by thrombin but were fully activated by the agonist peptide TFRIFD (data not shown). METHODS. The *Xenopus* thrombin receptor cDNA was cloned by polymerase chain reaction (PCR) amplification¹⁶ of *Xenopus* genomic DNA

		CLEAVAGE RECOGNITION SEQUENCE	TETHERED LIGAND/ AGONIST PEPTIDE DOMAIN	HIRUDIN- LIKE DOMAIN	
X 1	MMELRVLLLLLLTLLGAMGSLCLANS	MTIK /	TFRIFD	DSE	SEFEEI
H 1	_GPR_L__VAACFS_C_PLL_ARTRARRPES_ATNAT	LDPR /	S_LLRN	PN	DKY_PF
X 58	PW DEL DESGEGSGDQAPVRSAR	KPIRRNITKEAQ	TM1	YLSSQWLTKFVPSLYTVVFIIVGLP	
H 56	WE__EKN__LTEYRLVSINK_SPLQ_QLPAF_SED_SG		--T_S__L__V__G__V_S__		
X 118	LNLLAIIFLFKMKVR	TM2	EXTRACELLULAR LOOP 1	IAYHLSGNDWLFPGG	
H 119	_IM_VV_IL_K			_S_YF__S__Q__SE	
X 173	MCRIVTAIFYCNMYCSVLLIASI	SVDRFLAVVYPMHLSQSWRTMSRAY	TM3	MACSFIWLISIASTIPLLV	
H 174	L_F__A__A_I_MTV_	I__			

using degenerate oligonucleotide primers based on sequences in intracellular loop 1 and transmembrane domain 7 conserved in the human, hamster, mouse and rat thrombin receptor homologues. This PCR product was used to screen a *Xenopus* brain cDNA library (gift from N. Schneider and R. Axel). The nucleotide sequence of the resulting full-length cDNA was deposited in Genbank (accession number pending).

FIG. 2 The thrombin receptor's extracellular surface defines agonist specificity. Concentration-response curves for the *a*, human, or *b*, *Xenopus* agonist peptides acting at the human and *Xenopus* thrombin receptors and the human outside (HO) receptor chimaera. The human and *Xenopus* agonist peptides were the carboxamide forms of SFLLRN and TFRIFD, respectively, and were purified by reverse-phase HPLC before use. Wild-type (HWT' and XWT') and chimaeric HO thrombin receptor cRNAs were generated and expressed in *Xenopus* oocytes and agonist-induced ^{45}Ca release determined as described (Fig. 3). The HWT' and XWT' concentration-response curves represent 10 separate experiments with duplicate determinations at each agonist concentration; the HO curve represents three experiments. Data are expressed as per cent maximum response $\pm$ s.d. Maximum responses were typically a 30–50-fold increase in ^{45}Ca release over basal and were comparable for HWT', XWT' and HO.



and 5 in both agonist peptides contribute substantially to agonist peptide specificity (Table 1). Taken together with the receptor chimaera data, these observations imply that the human and *Xenopus* thrombin receptors are an example of complementary mutations evolving within a single molecule. Specifically, changes within the receptors' extracellular amino-terminal exodomain (residues 76–93) and second extracellular loop (residues 244–268) accommodate differences in the agonist peptide domain at positions 4 and 5. Human–*Xenopus* chimaeric receptors cannot be used to find out where structures common to both agonist peptides interact with the receptor. Docking of such structures (that is, the F2 side chain, protonated N terminus, and main chain) with either surface or transmembrane domains is plausible.

In summary, these studies show that the extracellular surfaces of the thrombin receptor determine the receptor's specificity for the human rather than *Xenopus* agonist peptides. In the well studied adrenergic receptor and rhodopsin systems, agonists or retinal interact with residues believed to lie exclusively within the receptor's transmembrane domains¹. The extraordinary gain of responsiveness to the human thrombin receptor's agonist peptide and concomitant loss of responsiveness to the *Xenopus* peptide caused by replacing the *Xenopus* receptor's extracellular surface with that of the human receptor strongly suggests that these peptide agonists cause signalling, at least in part, by interacting with surface rather than transmembrane structures. The dependence of agonist binding on particular surface residues in the neurokinin receptors^{2–5} and the receptors for growth-horm-

FIG. 3 Receptor domains mediating thrombin receptor agonist peptide specificity. Wild-type or chimaeric receptors were expressed in *Xenopus* oocytes and EC_{50} s determined for agonist-induced calcium mobilization by human or *Xenopus* agonist peptides, SFLLRN or TFRIFD, respectively. Mutant receptors cDNAs were produced by standard oligonucleotide-directed mutagenesis and subcloning techniques^{17,18}, then subcloned into pFROG⁸. Capped cRNAs were generated and microinjected into *Xenopus* oocytes⁸. Relative levels of surface expression of wild-type and mutant receptors were determined after 24 h as binding of monoclonal antibody to the DYKDDDD epitope, as described^{14,19}. For each construct, the amount of cRNA injected per oocyte was adjusted such that the level of surface expression was comparable for the receptors tested; the amount of cRNA injected for different constructs ranged from 12.5 to 25 ng per oocyte. HWT', human wild type receptor with the DYKDDDD epitope introduced at its N terminus as described¹⁴, with native human sequence from residue 36 onwards; XWT', *Xenopus* wild-type receptor with DYKDDDD epitope introduced at its N terminus analogous to HWT', with native *Xenopus* sequence from residue 37 onwards. HWT' and XWT' had agonist peptide specificities identical to their respective wild-type receptors (data not shown) and were used because of the need to quantitate expression on the oocyte surface. Chimaeric receptors contained native *Xenopus* sequence replaced with human amino-acid sequence as indicated: HAT75, HWT' to residue 75; HAT93, HWT' to human residue 93; HECL1, human residues 160–174; HECL2, human

cRNA Expressed	Construct	EC_{50} (μM)	
		Human SFLLRN	Xenopus TFRIFD
1. Human thrombin receptor	1.	0.2	10
2. <i>Xenopus</i> thrombin receptor	2.	>300	0.3
3. Human outside (HO) receptor	3.	0.8	300
4. Human amino terminus to 75 (HAT75)	4.	200	0.3
5. Human amino terminus to 93 (HAT93)	5.	20	6
6. Human extracellular loop 1 (HECL1)	6.	>300	0.3
7. Human extracellular loop 2 (HECL2)	7.	10	1
8. Human extracellular loop 3 (HECL3)	8.	>300	30
9. HAT93 plus HECL2	9.	0.1	5

residues 244–268; HECL3, human residues 333–349. HO contained the HAT93 and HECL1, 2 and 3 substitutions. Agonist-induced ^{45}Ca release was determined as described⁸. Concentration-response for curves for HWT', XWT', and each chimaera with both agonist peptides were run in parallel in each experiment. EC_{50} s were estimated from concentration-response curves that included responses to four concentrations of agonist spanning each construct's EC_{50} and reached saturation as shown in Fig. 2. Maximal responses expressed as fold increase in ^{45}Ca release were typically 30–50 fold and were comparable for the various constructs. Each result was replicated at least three times.

one-releasing factor⁶ suggest that this paradigm may operate in many peptide receptors. □

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1. Dohman, H. G., Thorner, J., Caron, M. G. & Lefkowitz, R. J. *A. Rev. Biochem.* **60**, 651–688 (1992).
2. Fong, T. M., Huang, R.-R. C. & Strader, C. D. *Biochemistry* **31**, 11806–11811 (1992).
3. Fong, T. M., Huang, R.-R. C. & Strader, C. D. *J. biol. Chem.* **267**, 25664–25667 (1992).
4. Gether, U., Johansen, T. E. & Schwartz, T. W. *J. biol. Chem.* **268**, 7893–7898 (1993).
5. Gether, U. *et al. Nature* **362**, 345–348 (1993).
6. Lin, S.-C. *et al. Nature* **364**, 208–213 (1993).
7. Fathi, Z., Benya, R. V., Shapira, H., Jensen, R. T. & Battey, J. F. *J. biol. Chem.* **268**, 14622–14626 (1993).
8. Vu, T.-K. H., Hung, D. T., Wheaton, V. I. & Coughlin, S. R. *Cell* **64**, 1057–1068 (1991).
9. Vu, T.-K. H., Wheaton, V. I., Hung, D. T. & Coughlin, S. R. *Nature* **353**, 674–677 (1991).
10. Fenton, J. W. *Sem. Hemostas. Thrombos.* **14**, 234–240 (1988).
11. Coughlin, S. R., Vu, T.-K. H., Hung, D. T. & Wheaton, V. I. *J. clin. Invest.* **89**, 351–355 (1992).
12. Vassallo, R. R. J., Kieber-Emmons, T., Cichowski, K. & Brass, L. F. *J. biol. Chem.* **267**, 6081–6085 (1992).
13. Scarborough, R. M. *et al. J. biol. Chem.* **267**, 13146–13149 (1992).
14. Ishii, K., Hein, L., Kobilka, B. & Coughlin, S. R. *J. biol. Chem.* **268**, 9780–9786 (1993).
15. Rydel, T. J. *et al. Science* **249**, 277–280 (1990).
16. Saiki, R. K. *et al. Science* **230**, 1350–1354 (1985).
17. Sambrook, J., Fritsch, E. F. & Maniatis, T. *Molecular Cloning* (Cold Spring Harbor Press, New York, 1989).
18. Kunkel, T. A., Roberts, J. D. & Zakour, R. A. *Meth. Enzym.* **154**, 367–382 (1987).
19. Lanza, F. *et al. J. biol. Chem.* **266**, 10638–10645 (1991).

Calmodulin interacts with amphiphilic peptides composed of all D-amino acids

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CALMODULIN binds to amphiphilic, helical peptides of a variety of amino-acid sequences^{1–4}. These peptides are usually positively charged, although there is spectroscopic evidence that at least one neutral peptide binds⁵. The complex between calmodulin and one of its natural target peptides, the binding site for calmodulin on smooth muscle myosin light-chain kinase (RS20)⁹, has been investigated by crystallography¹⁰ and NMR^{11–13} which have characterized the interactions between the ligand and the protein. From these data, it appears that the calmodulin-binding surface is sterically malleable and van der Waals forces probably dominate the binding. To explore further this apparently permissive binding, we investigated the chiral selectivity of calmodulin using synthesized analogues of melittin and RS20 that consisted of only D-amino acids. Fluorescence and NMR measurements show that D-melittin and D-RS20 both bind avidly to calmodulin, probably in the same general binding site as that for peptides having all L-amino acids. The calmodulin–peptide binding surface is therefore remarkably tolerant sterically. Our results suggest a potentially useful approach to the design of non-hydrolysable or slowly hydrolysable intracellular inhibitors of calmodulin.

The unbound D-form of each peptide (MLT and RS20) displayed fluorescence properties similar to the L-form, including emission maxima, quantum yields, anisotropies and lifetimes. The far-ultraviolet circular dichroism (CD) of the D-peptides yielded positive mirror images of the solution spectra of the L-

peptides (Fig. 1). The enantiomers also showed identical one-dimensional ¹H-NMR spectra and they co-eluted on HPLC.

The fluorescence of the single tryptophan (Trp) residue in each peptide is used to monitor the interaction of these amphiphilic peptides with calmodulin (CAM) (CAM contains no Trp). Figure 2 illustrates the fluorescence response of D- and L-melittin and RS20 upon interaction with CAM. Binding resulted in marked changes in fluorescence emission spectra, quantum yields (expressed simply as fluorescence intensities) and anisotropy (r_{ss}). The emission spectral changes may be attributed to a partial sequestration of the indole moiety away from water. An increase in the steady state anisotropy is due, in part, to the substantially larger molecular mass of the CAM–peptide complex when compared to that of the peptide alone.

We calculated the binding stoichiometry of melittin (MLT) with CAM using the Job plot¹⁴. Normalized anisotropy was calculated as $(A(x) - A_0)/(A_{im} - A_0)$, where $A(x)$ is the sample anisotropy, A_0 is the anisotropy of free melittin in buffer, A_{im} is the maximum anisotropy of melittin in the presence of excess

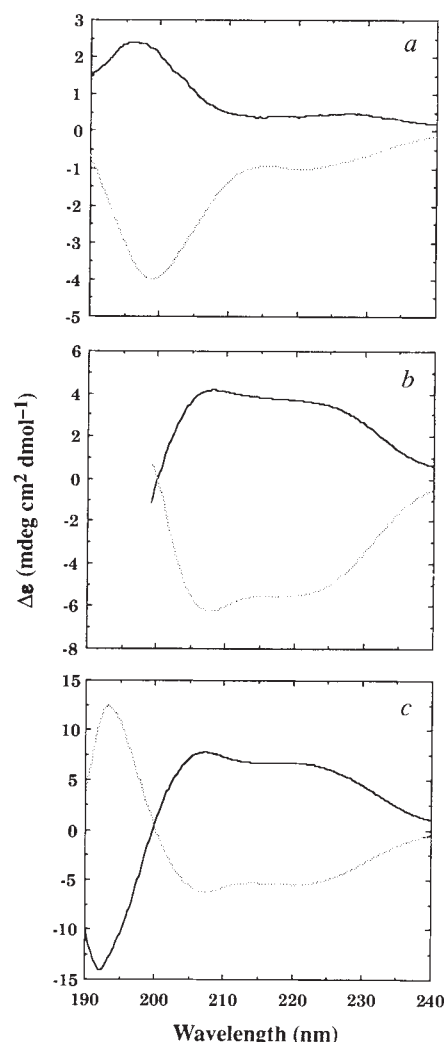


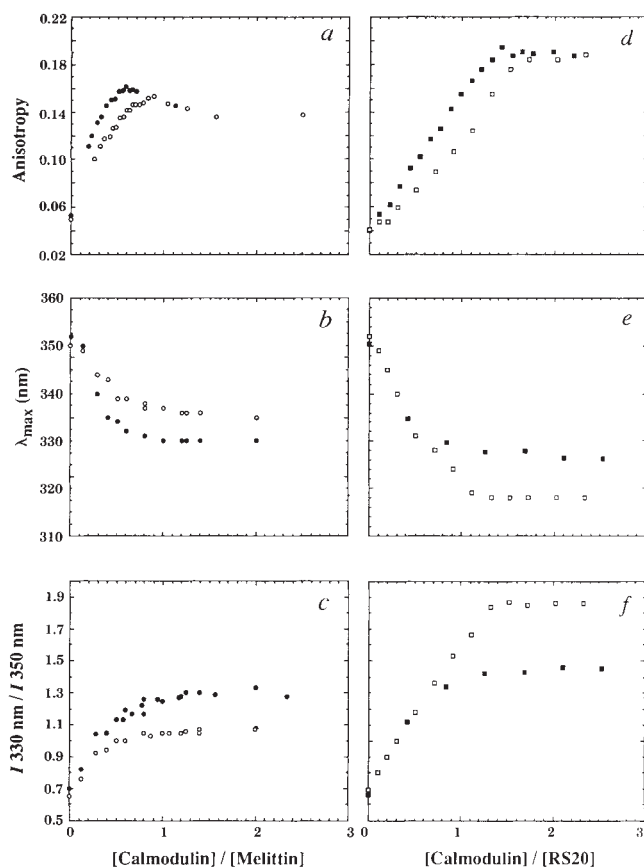
FIG. 1 Far-UVCD spectra of D (full line) and L (dotted line) melittin in different solution conformations: a, in water, monomeric random coil, b, in 0.8 M phosphate, α -helical tetramer; c, in 90% methanol, monomeric α -helix^{25,26}.

METHODS. Circular dichroism was measured on a Jasco J500A spectropolarimeter interfaced through the IF500 to an IBM PS/2 model 30. Scanning parameters were: 0.2 nm interval, 1.0 nm spectral bandwidth, 8-s time constant, 10 nm min⁻¹, with 10 accumulations using a 0.1-cm light path. The instrument was calibrated with camforsulphonic acid and with androsterone to <2% error in amplitude.

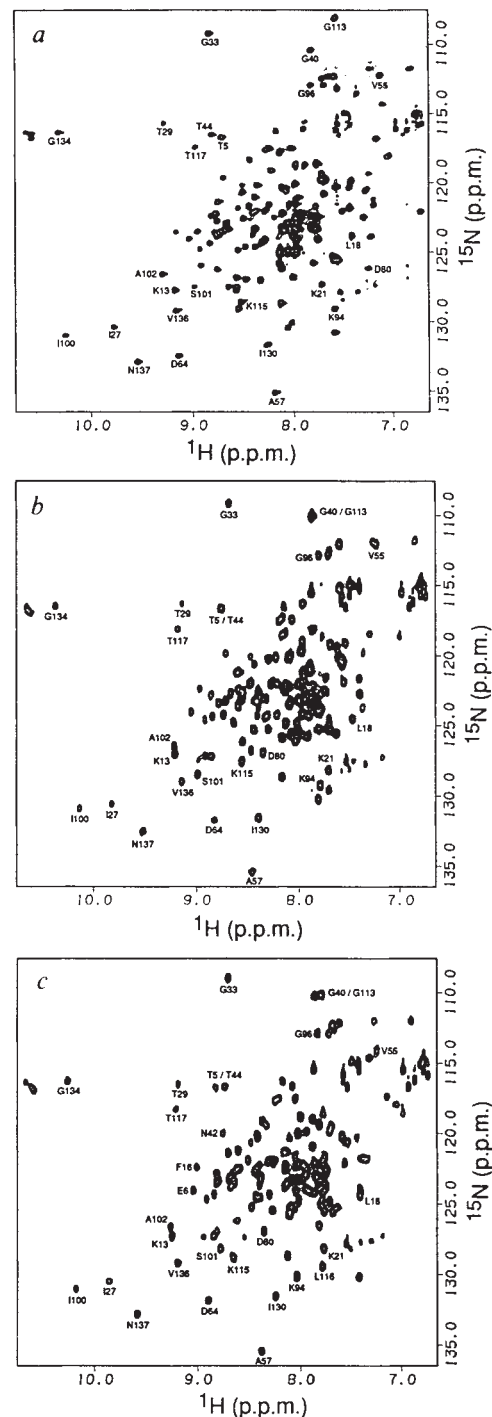
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Far-ultraviolet CD provided additional evidence for binding

Far-ultraviolet CD provided additional evidence for binding



METHODS. Purified VU-1 calmodulin was expressed from a synthetic calmodulin gene⁹. The 100 μ M stock solutions of CAM and peptides were dissolved in 120 mM KCl, 20 mM MOPS (or TES) buffer, 0.25 mM CaCl_2 , pH 7.0. Anisotropy measurements were made on an SLM 4800 fluorescence spectrophotometer, exciting at 300 nm with a 1-nm band pass and with an emission filter (WG345). Fluorescence emission spectra were collected on a Perkin-Elmer fluorimeter (model MPF-66) and were corrected for instrumental artefacts from 200 to 600 nm. Peptides were synthesized by Fmoc strategy using an Applied Biosystems 430A or 431A automated synthesizer (Foster City, CA). Protocols and reagents used were as recommended by the manufacturer. Peptides were purified by reverse-phase HPLC on a Vydac 2.2 cm $\times$ 25 cm preparative column (The Separation Group, Hesperia, CA) using a trifluoroacetic acid/acetonitrile solvent system. Peptide integrity was monitored by amino-acid analysis or mass spectroscopy.

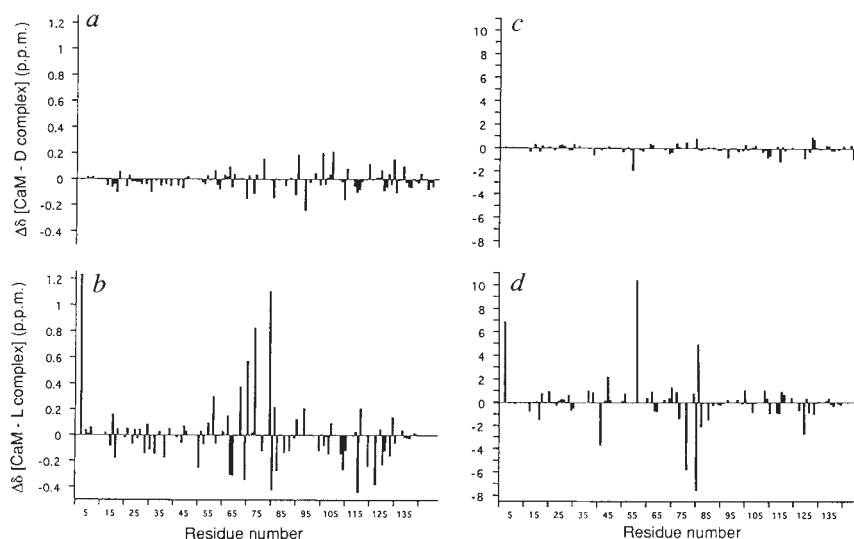


METHODS. Uniformly ^{15}N -enriched CAM was prepared essentially as described¹². NMR samples were prepared with ^{15}N -CAM at 2.0 mM (a) and 0.8 mM (b and c) in 10 mM imidazole- d_4 (imidazole deuterated in four positions), 40 mM CaCl_2 buffer, pH 6.5, in 90% $\text{H}_2\text{O}/10\%$ D_2O . HSQC spectra²⁷ were collected at 298 K with a ^1H frequency of 500.139 MHz. 128 hypercomplex points²⁸ of 32 scans per free induction decay were acquired in t_1 . Sweep widths of 6,666.7 Hz and 2,016 Hz were used for the hydrogen and nitrogen dimensions and processed to give a final digital resolution of 3.2 and 2.0 Hz per point, respectively.

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FIG. 4 Comparison of the chemical shift changes of backbone amide ^1H and ^{15}N resonances of CAM produced upon binding of L-RS20 (b and d) and D-RS20 (a and c). Shown are the chemical shift changes of CAM amide ^{15}N resonances which occur upon binding of the L-RS20 peptide (d) and the D-RS20 peptide (c). Also shown are the chemical shift changes of CAM amide ^1H resonances seen upon binding of the L-RS20 peptide (b) and the D-RS20 peptide (a).

METHODS. The L-RS20-CAM complex was assigned by reference to resonance assignments of a homologous complex¹². The D-RS20:CAM complex was assigned by reference to, by means of fast-exchange averaging, the spectrum of free-calcium-saturated CAM²⁵. A total of 10 titration points were used.



of the peptides to CAM, as well as demonstrating that the origin of helical changes occurring during complex formation is principally from the peptide rather than the CAM. These CD results are striking in that the increase in (negative) ellipticity evident on binding of L-MLT is of the same magnitude as the decrease in ellipticity observed when D-MLT. Available NMR and X-ray diffraction data have confirmed that the average overall helicity of CAM changes little upon binding peptide.

Multinuclear and multidimensional NMR techniques were used to examine further the similarity of the binding sites for the L-RS20 and D-RS20 peptides on CAM. As with homologous peptides^{15,16}, the L-RS20-CAM complex is in slow exchange with its dissociated components (Fig. 3). The 'off' rate can be estimated to be less than 1 s^{-1} . In contrast, the D-RS20 peptide is in fast exchange on the NMR chemical shift timescale, with the 'off' rate being of the order of 10^2 s^{-1} . As the D-RS20 peptide has similar affinity for calcium-loaded CAM, the increased 'off' rate requires that the 'on' rate for the D-peptide is also increased and approaches that expected for diffusion-limited binding.

The presence of fast exchange allowed the cross assignment of the D-RS20-CAM complex directly from the titration of CAM with peptide (not shown). Complex formation of either peptide with CAM results in changes in the chemical shifts of the backbone amide ^{15}N and ^1H resonances throughout the protein, indicating that both peptides perturb the structures of N-terminal and C-terminal calcium-binding domains as well as the connecting central helix (Fig. 4). Although both peptides appear to interact with similar regions of CAM, their spectra reveal that the magnitude of CAM chemical shift changes upon complex formation is much greater for L-RS20 than for D-RS20 (Fig. 4).

These data are consistent with a model for the binding of D-RS20 in which the peptide alternately interacts with each of the globular domains of CAM, without causing the collapse of the complex, to form the compact globular state reached by the L-peptide complex^{12,17,18}. Such an interaction would be consistent with rapid on and off rates and with the smaller chemical shift changes observed upon binding of the D-RS20 peptide relative to that seen for binding of the L-RS20 peptide. Furthermore, the amide hydrogens of the two β -sheet regions of CAM (residues 25–29, 61–65 and 98–102, 134–138) in the L-RS20 complex show a trend of downfield shifting relative to the D-RS20 complex. Such downfield chemical shifts have been associated with increasing hydrogen-bond strength (shorter distance) in β -sheet structures in a homologous L-peptide complex¹⁵.

Our results show that not only does the CAM-peptide binding surface behave as though it is achiral, but given the mirror-image

side-chain orientations in the enantiomeric peptides, the binding surface of CAM must also be sterically malleable and less dependent on specific charge-charge interactions.

The possible chiral sensitivity of the structure and interaction of peptide-macromolecule complexes has been explored extensively, for example in protein-lipid systems¹⁹ and in protein-ligand signalling^{20–23}, with positive and negative results. But the strong interaction revealed here of a naturally occurring L-protein with the D-enantiomer of 20- and 26-residue peptides is, to our knowledge, unprecedented. The all-D forms of melittin and RS20 might be effective inhibitors of CAM function: they should be resistant to degradation by the usual proteases^{22,24} and so would be useful long-lived intracellular inhibitors. Also, other proteins that bind a range of peptide ligands, particularly the major histocompatibility complex, might be able to bind peptides with all D-amino acids. □

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1. Erickson-Viitanen, S. & DeGrado, W. F. *Meth. Enzym.* **139**, 455–478 (1987).
2. O'Neil, K. T. & DeGrado, W. F. *Trends biochem. Sci.* **15**, 59–64 (1990).
3. Comte, M., Maulet, Y. & Cox, J. A. *Biochem. J.* **209**, 269–272 (1983).
4. Malenik, D. A. & Anderson, S. R. *Biochemistry* **23**, 2420–2427 (1984).
5. Cox, J. A., Comte, M., Fitton, J. E. & DeGrado, W. F. *J. biol. Chem.* **260**, 2527–2534 (1985).
6. Maulet, Y. & Cox, J. A. *Biochemistry* **22**, 5680–5686 (1983).
7. McDowell, L., Sanyal, G. & Prendergast, F. G. *Biochemistry* **24**, 2979–2984 (1985).
8. DeGrado, W. F., Prendergast, F. G., Wolfe, H. R. Jr & Cox, J. A. *J. Cellbiochem.* **29**, 83–93 (1985).
9. Lukas, T. J., Burgess, W. H., Prendergast, F. G., Lau, W. & Watterson, D. M. *Biochemistry* **25**, 1458–1464 (1986).
10. Meador, W. E., Means, A. R. & Quiocho, F. A. *Science* **257**, 1251–1255 (1992).
11. Ikura, M., Kay, L. E., Krinks, M. & Bax, A. *Biochemistry* **30**, 5498–5504 (1991).
12. Roth, S. M. et al. *Biochemistry* **31**, 1433–1451 (1992).
13. Seeholzer, S. H. et al. in *Calcium Binding Proteins in Health and Disease* 360–371 (1987).
14. Huang, C. Y. *Meth. Enzym.* **87**, 509–525 (1982).
15. Seeholzer, S. H. & Wand, A. J. *Biochemistry* **28**, 4011–4020 (1989).
16. Roth, S. M. et al. *Biochemistry* **30**, 10078–10084 (1991).
17. Ikura, M. et al. *Science* **256**, 632–638 (1992).
18. Meador, W. E., Means, A. R. & Quiocho, F. A. *Science* **257**, 1251–1254 (1992).
19. Wade, D. et al. *Proc. natn. Acad. Sci. U.S.A.* **87**, 4761–4765 (1990).
20. Nomizu, M. et al. *J. biol. Chem.* **267**, 14118–14121 (1992).
21. Tomita, U. et al. *Biochem. biophys. Res. Commun.* **178**, 400–406 (1991).
22. deL. Milton, R. C., Milton, S. C. F. & Kent, S. B. H. *Science* **256**, 1445–1448 (1992).
23. Corigliano-Murphy, M. A. et al. *Int. J. Pept. Prot. Res.* **25**, 225–231 (1985).
24. Dintzis, H. M., Symer, D. E., Dintzis, R. Z., Zawadzke, L. E. & Berg, J. M. *Proteins* **16**, 306–308 (1993).
25. Terwilliger, T. C., Weissman, L. & Eisenberg, D. *Biophys. J.* **37**, 353–361 (1982).
26. Terwilliger, T. C. & Eisenberg, D. *J. biol. Chem.* **257**, 6016–6022 (1982).
27. Bax, A., Ikura, M., Kay, L. E., Torchia, D. A. & Tschudin, R. J. *magn. Res.* **86**, 304–318 (1982).
28. Marion, D., Ikura, M., Tschudin, R. & Bax, A. J. *magn. Res.* **85**, 393–399 (1989).
29. Ikura, M., Kay, L. E. & Bax, A. *Biochemistry* **29**, 4659–4669 (1990).

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Bacteriophage T4 encodes a co-chaperonin that can substitute for *Escherichia coli* GroES in protein folding

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SEVERAL bacteriophages use the *Escherichia coli* GroES and GroEL chaperonins for folding and assembly of their morphogenetic structures¹. Bacteriophage T4 is unusual in that it encodes a specialized protein (Gp31) that is thought to interact with the host GroEL and to be absolutely required for the correct assembly of the major capsid protein (Gp23) *in vivo*²⁻⁴. Here we show that despite the absence of amino-acid sequence similarity between Gp31 and GroES^{5,6}, Gp31 can functionally substitute for the GroES co-chaperonin in the morphogenesis of bacteriophages λ and T5, the *in vivo* and *in vitro* chaperonin-dependent assembly of ribulose biphosphate carboxylase (Rubisco), as well as overall bacterial growth at the non-permissive temperature. Like GroES, the bacteriophage Gp31 protein forms a stable complex with the *E. coli* GroEL protein in the presence of Mg-ATP and inhibits the ATPase activity of GroEL *in vitro*.

To determine whether Gp31 functions as a co-chaperonin with GroEL, its ability to substitute for GroES in the morphogenesis of other bacteriophages was examined using three different *E. coli* *groES* mutants⁷ (Table 1): G24D and G23D, in which a glycine is replaced by an aspartic acid at positions 24 and 23, respectively, and A31V, which contains an alanine to valine substitution at position 31. All three mutations block the assembly

of the head structure of bacteriophage λ , whereas the G24D and G23D mutations block the assembly of bacteriophage T5 tails as well⁷. The various *groES* mutant strains were transformed with either plasmid pBAD22 (vector), pS4 (*groES*⁺)⁸ or pSV25 (gene 31⁺) and examined for their ability to support the growth of λ and T5. In the presence of either plasmid-encoded GroES or Gp31, both bacteriophages were able to form plaques on the *groES* mutant strains, showing that bacteriophage T4 Gp31 can effectively substitute for GroES in the morphogenesis of bacteriophages λ and T5 (Table 1). Although the growth of bacteriophage T5 on the G24D or G23D mutant is similar in the presence of either pS4 (*groES*⁺) or pSV25 (gene 31⁺), growth of bacteriophage λ on all three *groES* mutants is more efficient in the presence of pS4 than with pSV25.

To determine whether Gp31 could also replace GroES in general cell growth, the G23D *groES* mutant was used because it is unable to form colonies at 43 °C (ref. 7). Serial dilutions of G23D cells transformed with either pS4 (*groES*⁺) or pSV25 (gene 31⁺) had a similar number of colony-forming units when grown at 43 °C, although the Gp31-carrying colonies were smaller (Fig. 1a). Colonies were not observed at 43 °C with vector-transformed cells. These results show that Gp31 is able to suppress the temperature-sensitive growth defect, and probably can act as a co-chaperonin during the folding of a wide range of cellular proteins, because most of them interact with GroEL^{9,10}. Similar results were obtained with the G24D mutant, which also exhibits a 'temperature-sensitive' phenotype at 43 °C (data not shown).

As a specific example of cellular protein assembly, the influence of Gp31 on the assembly of the cyanobacterial hexadecameric Rubisco enzyme in *E. coli* was investigated. The assembly of Rubisco requires both GroEL and GroES, because the assembly of a functional protein is greatly diminished in either *groEL* or *groES*-defective cells¹¹. A dual plasmid system was used to examine Rubisco assembly in the G24D *groES* mutant. The plasmid pRUB6 contains the Rubisco *rbcl*⁺ and *rbcS*⁺ structural genes, encodes resistance to chloramphenicol, and is compatible with the second ampicillin-resistance-encoding plasmid introduced, namely pBAD22 (vector), or its derivatives, pSV25

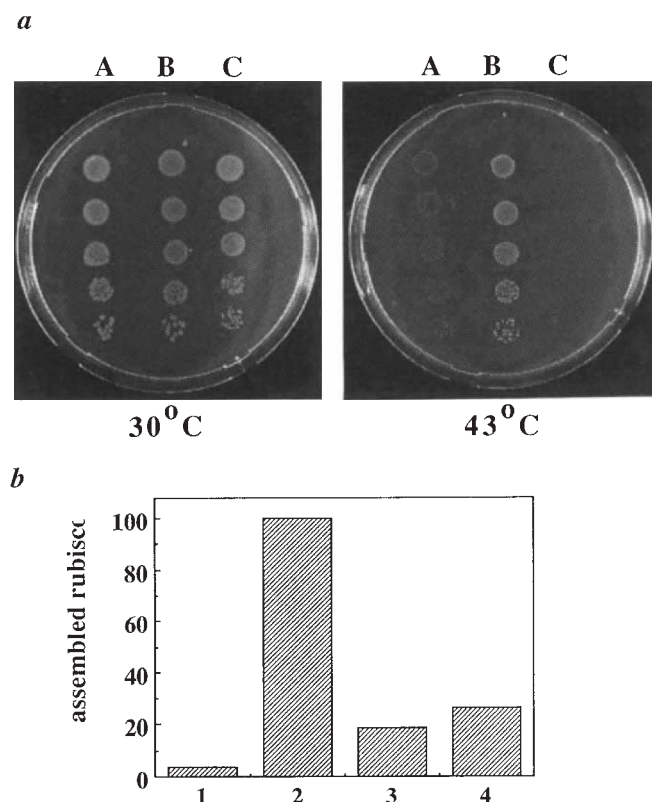


FIG. 1 Suppression of growth and protein folding defects in *groES* mutant strains by bacteriophage T4 gene 31. *a*, Suppression of the temperature-sensitive phenotype of *E. coli* *groES* G23D mutant by overproduction of Gp31; A, pSV25 (gene 31⁺); B, pS4 (*groES*⁺); C, pBAD22 (vector). *b*, Rubisco assembly in the *E. coli* *groES* mutant G24D. Rubisco activity is expressed as a percentage, where 100% is the value obtained for pOF39 and pRUB6; 1, pBAD22 (vector); 2, pOF39 (*groES*⁺, *groEL*⁺); 3, pS4 (*groES*⁺); 4, pSV25 (gene 31⁺). In addition, all strains contained the plasmid pRUB6 (*rbcl*⁺, *rbcS*⁺).

METHODS. *a*, The *E. coli* *groES* G23D mutant was transformed with either plasmid pBAD22 (vector), pS4 (*groES*⁺)¹² or pSV25 (gene 31⁺) and grown at 37 °C to an OD of 0.6 at 600 nm, in NZY* (as NZY but with 2.5 g NaCl l⁻¹) medium plus ampicillin (100 µg ml⁻¹). Aliquots of 10-fold serial dilutions were spot-tested onto NZY* plates containing ampicillin and 0.5% (w/v) arabinose. Plates were incubated at the indicated temperatures for 18 h. *b*, Plasmid pRUB6 was constructed by transferring the 2.2-kb *Pst*I fragment encoding the *A. nidulans* *rbcl*⁺ and *rbcS*⁺ genes, from pSV55¹⁶ into the *Pst*I site of pTG10¹¹. The Rubisco genes in pRUB6 are transcribed from the inducible *lacZ* promoter. Transformants of the *E. coli* *groES* G24D mutant carrying the plasmid pRUB6 and either plasmid pS4, pOF39⁸, pBAD22, or pSV25 (see legend to Table 1) were grown in NZY medium containing the appropriate antibiotics to an OD of 0.3 at 600 nm. After 2 h of additional growth in the presence of IPTG (1 mM) and arabinose (0.5% (w/v)), the cells were collected by centrifugation, washed and lysed by sonication at 0 °C in a solution containing 100 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 30 mM NaHCO₃, 15% (v/v) glycerol, 1 mM DTT, 1 mM PMSF and 1 mg ml⁻¹ of lysozyme. The protein concentration of the cell-free extract was adjusted to 5 mg ml⁻¹ and 100 µl was used to determine Rubisco activity¹⁷.

(gene 31⁺), or pS4 (*groES*⁺), or pOF39 (*groES*⁺, *groEL*⁺)⁸. Maximum Rubisco activity was detected when the entire *groE* operon (pOF39) was present, and designated as 100% (Fig. 1b, sample 2). When only the pBAD22 vector was present 4% Rubisco activity was detectable (Fig. 1b, sample 1). The Rubisco activity increased to about 20 or 30% when pS4 or pSV25 were present, respectively (Fig. 1b, samples 3 and 4). Both Gp31 and GroES are therefore able to increase the amount of assembled Rubisco in the G24D mutant strain, although not to the same extent as when the complete *groE* operon is present on a plasmid (pOF39). This differential amount of Rubisco activity is most probably related to an imbalance in the molar amounts of GroEL and either GroES or Gp31.

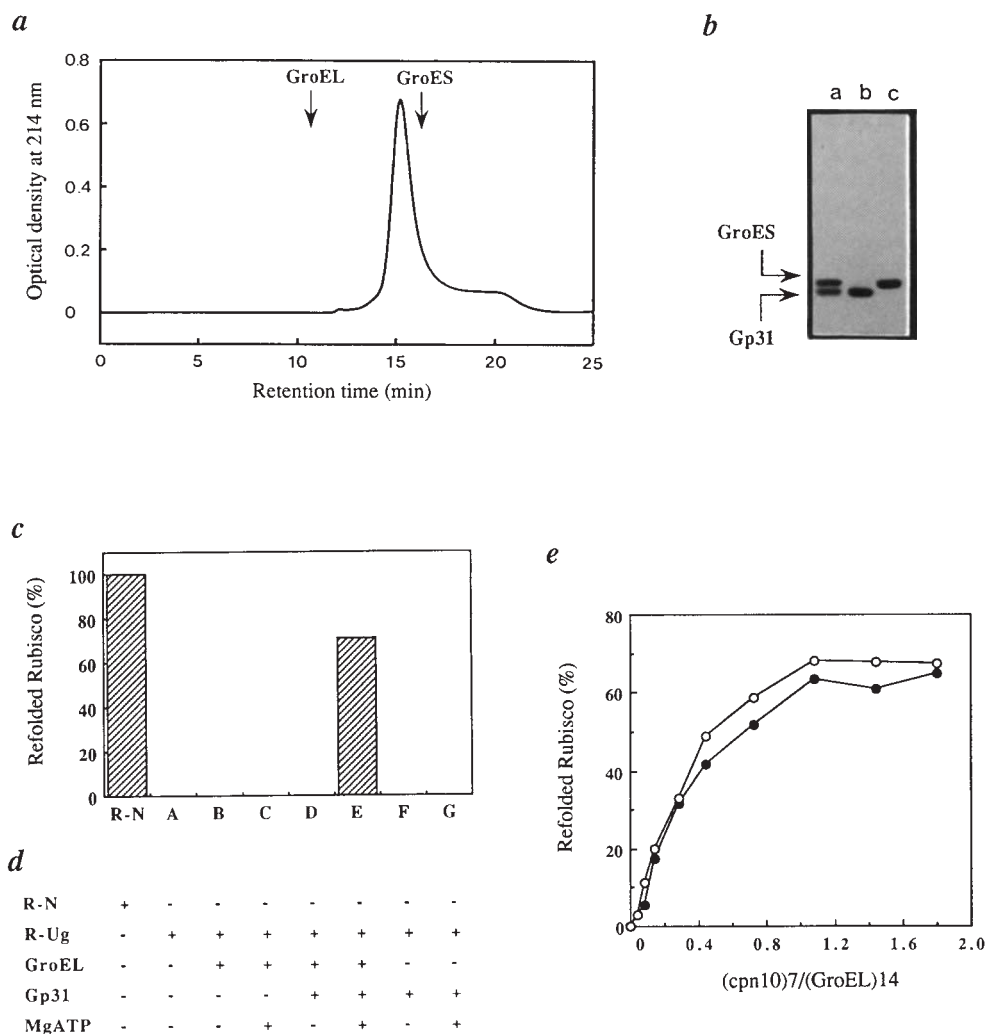
Evidence that Gp31 functions as a co-chaperonin *in vitro* was obtained in the following experiments. Gp31 was purified to homogeneity and was shown by gel filtration chromatography to form an oligomer of *M_r* 90K consisting of 10–12K subunits,

indicative of a heptameric structure (Fig. 2a, b). Examination of the conditions required for the chaperonin-dependent refolding of the chemically denatured dimeric Rubisco from *Rhodospirillum rubrum* showed that optimal reconstitution (70%) was obtained only in the presence of GroEL, Gp31 and Mg-ATP (Fig. 2c, d, sample E). When various amounts of either GroES or Gp31 were present in the Rubisco refolding assay, maximum refolding was observed with a 1:1 molar stoichiometry of Gp31 or GroES to GroEL (Fig. 2e).

These *in vitro* studies suggest a physical interaction between GroEL and Gp31, which was directly demonstrated by incubating the two proteins together in the presence or absence of Mg-ATP, and analysing the mixture by gel-filtration chromatography. In the presence of Mg-ATP, the GroEL fraction contained Gp31 (Fig. 3a, lane 2), but in the absence of the nucleotide Gp31 did not co-elute with GroEL (Fig. 3a, lane 1). The formation of a stable complex between GroEL and Gp31 in the pres-

FIG. 2 Purification and analysis of the bacteriophage T4-encoded Gp31 protein. **a**, Analysis of purified Gp31 by gel filtration chromatography. **b**, SDS-PAGE of Gp31 plus GroES (**a**), Gp31 (**b**) and GroES (**c**). **c**, Refolding of denatured *R. rubrum* Rubisco in the presence of GroEL, Gp31 and Mg-ATP. Rubisco activity is expressed as a percentage of the activity determined for an equal quantity of native Rubisco that had not been unfolded. **d**, Summary of the reaction conditions of the Rubisco refolding experiments shown in **c**, where R-N = native Rubisco and R-Ug = Rubisco unfolded in 6 M guanidine hydrochloride. **e**, Rubisco refolding in the presence of various concentrations of Gp31 (●) or GroES (○). Rubisco activity is expressed as under **d**.

METHODS. *E. coli* strain MC1009 containing plasmid pSV25 was grown in NZY medium (see legend to Table 1) at 37 °C to an absorbance of 0.8 at 600 nm. Synthesis of Gp31 was induced with 0.001% (w/v) arabinose for 16 h and the protein was purified using a modification of ref. 18. Gp31 was identified as a 10–12K Coomassie blue-stained band after SDS-PAGE (15%) (calculated *M_r* is 12,046; refs 5, 6). Ammonium sulphate precipitation (30 to 70% saturation) was followed by ion-exchange chromatography using a Q-Sepharose column. Gp31 was eluted with a 0–0.5 M NaCl gradient in 20 mM Tris-HCl pH 7.5, 1 mM EDTA and 1 mM β-mercaptoethanol. After gel filtration chromatography using a Superdex-200 column (Pharmacia) equilibrated in 100 mM Tris-HCl pH 7.6, the purified protein was stored at –80 °C in 50 mM Tris-HCl pH 7.5, 1 mM DTT, 0.1 mM EDTA and 10% (v/v) glycerol. The determined N-terminal amino acids at position 2 to 13 of the purified Gp31 showed 100% identity to the predicted sequence. **a**, An aliquot of purified Gp31 was applied onto a HPLC-TSK gel filtration column (type G3000SW, 75 × 600 mm) equilibrated in 100 mM Tris-HCl pH 7.6 and calibrated with purified GroEL, GroES, thyroglobin, gamma globulin, BSA, ovalbumin and myoglobin. **b**, Analysis of Gp31 by SDS-PAGE (15%) followed by staining with Coomassie blue. **c**, The chaperonin-dependent reconstitution of Rubisco was performed as initially described¹⁹. An aliquot of Rubisco that had



been denatured in a solution containing 6 M guanidine hydrochloride, 0.1 M Tris-HCl pH 7.6 and 0.1 mM DTT was diluted to a final concentration of 100 nM into a solution containing 50 mM Tris-HCl, pH 7.5, 6.7 mM MgCl₂, 9.5 mM KCl, 1.7 mM DTT, 3.1 μM BSA, 2.8 μM GroEL, 2.3 μM Gp31 and 2 mM Mg-ATP. After incubation at 23 °C for 60 min, the reaction was terminated using a hexokinase/glucose trap¹⁹ and the Rubisco activity was determined¹⁷. **e**, Essentially as described for **c**; the reaction mixtures contained 3.1 μM GroEL and various concentrations of either Gp31 (●) or GroES (○) (referred to as cpn10).

TABLE 1 Growth of bacteriophages λ and T5 on *groES* mutant strains

Amino-acid substitution in GroES*	Plasmid†	λ		T5	
		Burst size‡	Efficiency of plating§	Burst size	Efficiency of plating
G24D	pBAD22 (vector)	<0.01	<10 ⁻⁵	<0.01	<10 ⁻⁵
	pS4 (<i>groES</i> ⁺)	2.4	1.0	1.5	1.0
	pSV25 (gene 31 ⁺)	1.1	0.3	1.5	0.9
G23D	pBAD22 (vector)	<0.01	<10 ⁻⁵	<0.01	<10 ⁻⁵
	pS4 (<i>groES</i> ⁺)	2.0	1.0	0.9	1.0
	pSV25 (gene 31 ⁺)	0.8	0.5	0.6	1.0
A31V	pBAD22 (vector)	<0.01	2 × 10 ⁻⁵		
	pS4 (<i>groES</i> ⁺)	2.9	1.0		
	pSV25 (gene 31 ⁺)	1.2	0.4		

* The position and changed amino-acid residues in GroES due to the mutation are given using one-letter amino-acid code. For more information on mutant strains see ref. 7.

† The plasmid pS4 contains the *groE* promoter plus the entire *groES*⁺ gene⁸. Plasmid pSV25 was constructed by transferring the *EcoRI* and *SalI* fragment encoding the bacteriophage T4 gene 31 from pKL10344⁹ into pBAD22 (kindly provided by L.-M. Guzman-Verduzco and D. Belin). The gene 31 in pSV25 is transcribed from the arabinose promoter.

‡ The burst size for λ and T5 was determined at 37 °C¹⁵ on various *E. coli groES* mutant strains⁷ carrying the above plasmids. The strains were grown in NZY medium containing 10 g NZ amine, 5 g yeast extract and 5 g NaCl per l, pH 7.4 in the presence of 100 μ g ml⁻¹ ampicillin. Transcription of gene 31 was induced in the presence of 0.5% (w/v) arabinose. The titre of the bacteriophage solutions was determined using the *E. coli* permissive strain B178³ and the burst size is given as the number of viable bacteriophage progeny produced per infected bacterial cell.

§ The efficiency of plating of λ and T5 was determined at 37 °C on different *E. coli groES* mutant strains⁷ carrying the above plasmids and is reported as the number of plaques produced using a given plasmid in comparison to the number of plaques obtained with plasmid pS4. Strains were grown on NZY plates containing ampicillin and arabinose as described above.

|| Plaques with a diameter of ~0.2 mm; all other plaques had a diameter of ~1 mm.

ence of Mg-ATP is thus analogous to that observed between GroEL and GroES under identical conditions¹². The ATPase activity of GroEL is inhibited in the presence of GroES¹²⁻¹⁴. The addition of Gp31 inhibited the hydrolysis of ATP by GroEL, to the same extent as GroES (Fig. 3b).

The evidence presented here clearly demonstrates that bacteriophage T4-encoded Gp31 serves as a functional analogue of the bacterial GroES co-chaperonin. The absolute requirement of Gp31 for the correct assembly of the Gp23 capsid protein *in vivo* suggests that Gp31 possesses specific co-chaperonin properties that are absent in GroES. Alternatively, the growth of some

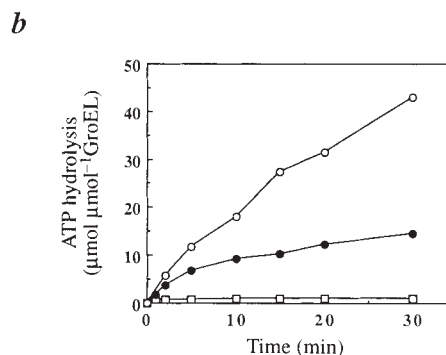


FIG. 3 Physical interaction between GroEL and Gp31. a, SDS-PAGE analysis. The proteins were stained with Coomassie blue. Samples with (2) and without (1) Mg-ATP were analysed after chromatography. Lane 3 represents purified Gp31. Arrows indicate the positions of GroEL and Gp31. b, ATPase activity of GroEL in the absence (○) or presence (●) of Gp31. A control reaction using identical concentrations of Gp31 alone is also shown (□).

METHODS. a, The formation of a binary complex between GroEL and Gp31 was as previously described for GroEL and GroES¹². The reaction mixture contained 80 mM Tris-HCl, pH 7.6, 8 mM MgCl₂, 8 mM KCl, 11.3 μ M GroEL and 15.7 μ M Gp31. Reaction mixture 2 also contained 0.6 mM Mg-ATP. The samples were incubated for 12 min at room temperature, cooled to 4 °C and loaded onto an HPLC-TSK sizing column in 100 mM Tris-HCl, pH 7.6, 10 mM MgCl₂ and 10 mM KCl. For reaction 2, the column buffer also contained 0.25 mM Mg-ATP. The proteins were eluted at 4 °C at a flow rate of 1.0 ml min⁻¹. Column fractions corresponding to the position of GroEL (10.5–11.5 min) were collected. Proteins were precipitated with acetone (80%) and analysed by SDS-PAGE (15%). b, ATP hydrolysis was measured as the release of radioactive inorganic phosphate¹². The reaction mixture contained 1 μ M GroEL in a solution of 40 mM Tris-HCl, pH 7.6, 4.6 mM MgCl₂, 4.6 mM KCl with (●) or without (○) 2 μ M Gp31.

viruses, such as T4, may impose great demands on the protein-folding machinery of the cell that can only be satisfied by the virus supplying some parts of the machinery. □

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- Zeilstra-Ryalls, J., Fayet, O. & Georgopoulos, C. A. *Rev. Microbiol.* **45**, 301–325 (1991).
- Laemmli, U. K., Gegin, F. & Gujer-Kellenberger, G. *J. molec. Biol.* **47**, 69–85 (1970).
- Georgopoulos, C. P. et al. *Nature New Biol.* **239**, 38–41 (1972).
- Takano, T. & Kakefuda, T. *Nature New Biol.* **239**, 34–37 (1972).
- Keppel, F. et al. *Gene* **86**, 19–25 (1990).
- Nivinskas, R. & Black, L. W. *Gene* **73**, 251–257 (1988).
- Landry, S. J. et al. *Nature* **364**, 255–258 (1993).
- Fayet, O., Louarn, J.-M. & Georgopoulos, C. *Molec. gen. Genet.* **202**, 435–445 (1986).
- Viitanen, P. V., Gatenby, A. A. & Lorimer, G. H. *Protein Sci.* **1**, 363–369 (1992).
- Horwich, A. L. et al. *Cell* **74**, 909–917 (1993).
- Goloubinoff, P., Gatenby, A. A. & Lorimer, G. H. *Nature* **337**, 44–47 (1989).
- Viitanen, P. V. et al. *Biochemistry* **29**, 5665–5671 (1990).
- Jackson, G. S. et al. *Biochemistry* **32**, 2554–2563 (1993).

- Todd, M. J., Viitanen, P. V. & Lorimer, G. H. *Biochemistry* **32**, 8560–8567 (1993).
- Sternberg, N. *J. molec. Biol.* **76**, 1–23 (1973).
- Gatenby, A. A., van der Vies, S. M. & Bradley, D. *Nature* **314**, 617–620 (1985).
- Lorimer, G. H., Badger, M. R. & Andrews, J. T. *Analyt. Biochem.* **78**, 66–75 (1977).
- Castillo, J. C. & Black, L. W. *J. biol. Chem.* **253**, 2132–2139 (1978).
- Goloubinoff, P., Christeller, J. T., Gatenby, A. A. & Lorimer, G. H. *Nature* **342**, 884–889 (1989).

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Structural and kinetic characterization of a β -lactamase-inhibitor protein

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THE past decade has seen an alarming worldwide increase in resistance to β -lactam antibiotics among many pathogenic bacteria¹, which is due mainly to plasmid- or chromosomally encoded β -lactamases that specifically cleave penicillin and cephalosporins, rendering them inactive. There is therefore a need to develop new strategies in the design of effective inhibitors of β -lactamase. All the small-molecule inhibitors in clinical use are not very effective and are rapidly degraded^{2,3}. Furthermore, newly characterized mutants of the plasmid-mediated β -lactamase TEM-1 are highly resistant to these small-molecule inhibitors, including clavulanic acid and tazobactam⁴. It has been shown that *Streptomyces clavuligerus* produces an exocellular β -lactamase inhibitory protein (BLIP; M_r 17.5 K)⁵. Here we present data defining BLIP as the most effective known inhibitor of a variety of β -lactamases, with K_i values in the subnanomolar to picomolar range. To identify

those features in BLIP that make it such a potent inhibitor, we have determined its molecular structure at 2.1 Å resolution. BLIP is a relatively flat molecule with a unique fold, comprising a tandem repeat of a 76-amino-acid domain. Each domain consists of a helix-loop-helix motif that packs against a four-stranded antiparallel β -sheet (Fig. 1a). To our knowledge, BLIP is the first example of a protein inhibitor having two similarly folded domains that interact with and inhibit a single target enzyme.

The molecular structure of BLIP was determined by the method of multiple isomorphous replacement (MIR; Fig. 1b). It has been refined using data in the resolution range of 20.0 Å to 2.1 Å to an agreement index ($R = \sum ||F_o| - |F_c|| / \sum |F_o|$, where $|F_o|$ and $|F_c|$ are the measured and calculated structure factor amplitudes, respectively) of 0.178 for 8,329 reflections with $|F_o| \geq \sigma |F_c|$. The resultant molecular model has good stereochemistry with root-mean-square (r.m.s.) deviations from ideality of 0.017 Å for bond lengths, 1.9° for bond angles, and 0.03 Å for planar groups. Representative electron density corresponding to a region of β -sheet in the molecule is shown in Fig. 1b.

The amino-acid sequence of BLIP shows significant internal identity that suggests an evolutionary history of gene duplication and fusion to give rise to the two-domain protein. The regions that exhibit the highest degree of internal sequence identity correspond to the helix-loop-helix motifs of each domain (Fig. 2a). The X-ray crystallographic model reveals that the structural similarity extends to the sheet regions as well. The two domains of BLIP can be defined from Ala 1 to Leu 76 and from Ala 80 to Val 165, with a short connecting tripeptide, Ala 77 to Ser 79 (Fig. 1a). Figure 2b shows the superposition of the two domains using the transformation generated by a least-squares minimization of the deviations in position of 56 common Ca atoms. These topographically equivalent regions comprise the residues that form the helix-loop-helix motif and segments of the four strands of antiparallel β -sheet in each domain.

The two domains of BLIP lie next to one another, resulting in an 8-stranded antiparallel β -sheet (Fig. 1a). In each domain, both helices run parallel to the strands of the sheet, forming

TABLE 1 Effect of BLIP on the activity of various β -lactamases and penicillin-binding proteins

β -lactamases*	Effect (K_i)†		
Group 1 enzymes		<i>E. coli</i> TEM-3	↓ (0.3 nM)
<i>E. coli</i> amp C	↑	<i>Proteus vulgaris</i> 1028	↓ (18 pM)
<i>Citrobacter freundii</i> 1203	↑	<i>Lysobacter enzymogenes</i>	↓ (ND)
<i>Enterobacter cloacae</i> 908R	—		
<i>E. cloacae</i> M6300	↑	Group 2c enzymes	
<i>Pseudomonas aeruginosa</i> 18SH	↑	<i>P. aeruginosa</i> PSE-1	↓ (3 nM)
<i>P. aeruginosa</i> 143724	—		
<i>P. aeruginosa</i> 143811R	—	Group 2d enzymes	
<i>Morganella morganii</i> V1627	—	<i>Salmonella typhimurium</i> OXA-2	—
<i>Providencia rettgeri</i> 5298	—		
Group 2a enzymes		Group 3 enzymes	
<i>S. aureus</i> 157	↑	<i>B. cereus</i> 5/B/6	—
<i>S. aureus</i> 853	↓ (3 μ M)	<i>Pseudomonas maltophilia</i>	—
<i>B. cereus</i> ATCC 1-27348	↓ (1 μ M)	<i>Aeromonas hydrophila</i>	—
<i>B. cereus</i> NCIB 8933	—		
<i>B. licheniformis</i> 749/C	↓ (3 μ M)	Penicillin binding proteins	
<i>Streptomyces albus</i> G	↓ (3 μ M)	<i>E. coli</i> PBP 1b	—
<i>Actinomadura</i> R39	↓ (11 μ M)	<i>E. coli</i> PBP 3	—
		<i>P. aeruginosa</i> PBP 3	—
Group 2b enzymes		<i>S. aureus</i> PBP 2'	—
<i>Klebsiella oxytoca</i> 1082	↓ (1 nM)	<i>Enterococcus faecalis</i> PBP 5	↓ (12 μ M)
<i>E. coli</i> TEM-1	↓ (0.6 nM)	<i>Streptomyces</i> R39 D α -carboxypeptidase	—
		<i>Streptomyces</i> R61 D α -carboxypeptidase	—

* β -Lactamases classified according to ref. 15.

† ↑, Stimulatory effect; ↓, inhibitory effect; —, no effect; ND, not determined. Enzyme inhibition assays were carried out using nitrocefin or imipenem as substrates in 50 mM sodium phosphate buffer, pH 7.0, after preincubation of the β -lactamases with various concentrations of BLIP at 30 °C. When inhibition was observed, K_i values (given in parentheses) were obtained from measurements of the initial rates of hydrolysis of the β -lactam substrates in the presence and absence of BLIP. The effect of BLIP on D α -carboxypeptidase activity was measured using the peptide substrate (Ac₂-L-Lys-D-Ala-D-Ala, where Ac is acetyl) and following the release of free D-Ala by the method in ref. 16. Other PBPs were assayed using nitrocefin as substrate to measure residual activity after pre-incubation with BLIP.

between them a small hydrophobic core. There is a disulphide bridge connecting the first two adjacent strands (β_1 – β_2 and β_5 – β_6) in each domain (Fig. 1a). Residues outside the topographically equivalent regions in the two domains of the molecule form loops of varying sizes that connect the four strands in the antiparallel β -sheet. The slight difference in size between the domains is largely due to a 10-residue insertion in the C-terminal domain. The segment Tyr 115 to Gly 124 extends strands β_5 and β_6 relative to β_1 and β_2 (Fig. 2a, b). The residues in the two equivalent loops connecting strands β_2 to β_3 and β_6 to β_7 are poorly ordered in the electron density map and correspond to the regions with the highest thermal parameters in the molecule.

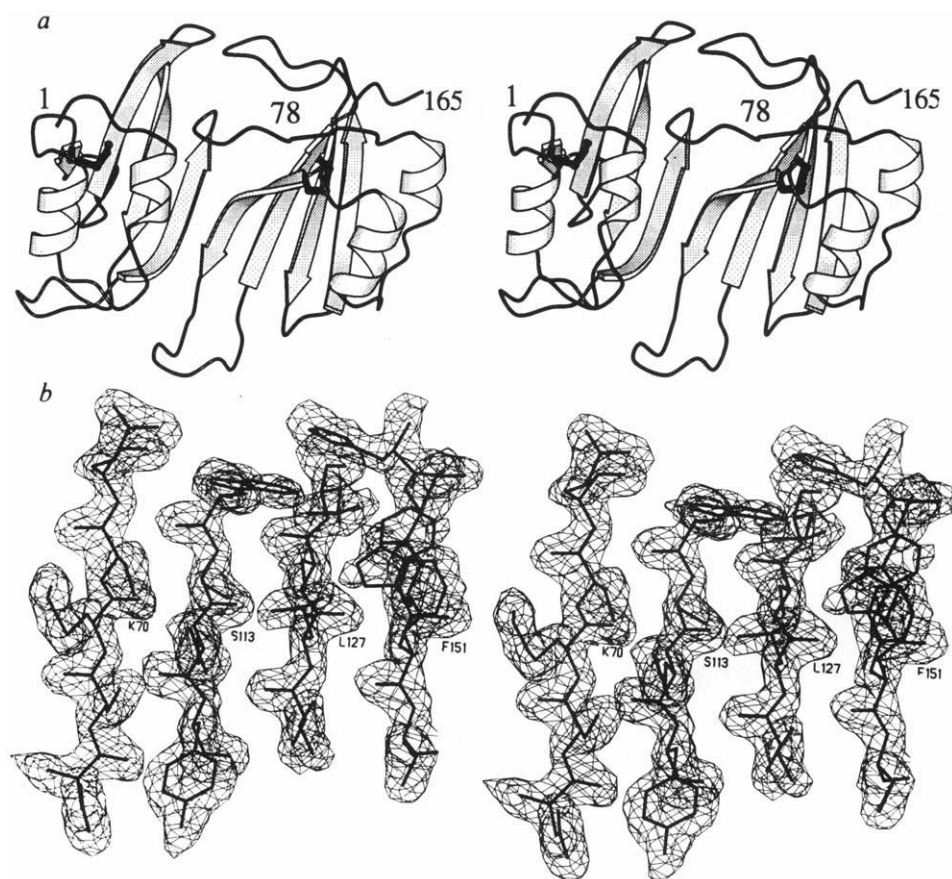
The range of enzymes affected by BLIP was investigated using a variety of β -lactamases and penicillin-binding proteins (PBPs). Table 1 summarizes the results, which show that BLIP resembles clavulanic acid (a small molecule that is a secondary metabolite from *S. clavuligerus*) in that it has no ability to inhibit group 1 or group 3 enzymes (see ref. 15 for classification of β -lactamases).

FIG. 1 a, Diagram showing regions of secondary structure in BLIP. These regions are defined using the criteria of Kabsch and Sanders in the program DSSP¹⁷. The molecule consists of two tandemly repeated domains each having a helix-loop-helix motif which is packed on one side of a four-stranded antiparallel β -sheet. There are three interdomain residues, Ala 77–Ser 79. Also shown are the two disulphide bridges in the molecule (Cys 30–Cys 42 in the N-terminal domain, and Cys 109–Cys 131 in the C-terminal domain) which are partly buried in the corresponding hydrophobic cores. The approximate dimensions of BLIP are $60 \times 35 \times 24$ Å. b, View of the final $2|F_o| - |F_c|$, α_c electron density distribution contoured at 2σ , with the refined molecular structure of BLIP superimposed. The region displayed encompasses His 148, which is surrounded by Trp 112, Trp 150 and Trp 162 (Fig. 3).

METHODS. The isolation and purification of BLIP has been described⁵. Crystals of BLIP were grown by vapour diffusion from solutions containing 10 mg ml^{-1} protein, 20% saturated $(\text{NH}_4)_2\text{SO}_4$ and 50 mM sodium citrate at pH 5.5. The crystals are monoclinic, space group C2, with unit cell dimensions of $a = 129.4$, $b = 26.2$, $c = 48.1$ Å, and a β -angle of 114.0° . Data for the native and three heavy-atom derivatives (1 mM K_2PtCl_4 , 1 mM mersalyl and 2 mM methyl mercury chloride) were collected on an SDMS twin area detector system¹⁸ using $\text{CuK}\alpha$ X-rays from a Rigaku RU200 generator running at 45 kV and 135 mA. Data processing was carried out with the software supplied¹⁹. For the initial native data set, 19,948 observations were merged to give a unique set of 7,166 reflections to 2.1 Å resolution (the merging R on intensities $(\sum \sum |I_j - \langle I \rangle| / \sum \langle I \rangle)$ was 0.035 for the native crystal). For this data set, the completeness of measurement to 2.5 Å is 95% and to 2.1 Å is 37%. Heavy-atom sites for the three heavy-atom derivatives were identified on difference Patterson maps computed at 3.5 Å resolution. Their positions, occupancies and B -factors were refined at 3.0 Å with the program PHARE (Z. Otwinoski, personal communication). The overall phasing power to 3.0 Å resolution for each of the derivatives was 1.75 (K_2PtCl_4), 1.74 (mersalyl) and 1.41 (methyl mercury chloride). The final overall figure of merit was 0.64 at this resolution. An electron density map computed using figure-of-merit weighted structure factor amplitudes

Unlike clavulanic acid, however, which uniformly inhibits all group 2 enzymes, BLIP shows a remarkable variability in its effect on these enzymes. All group-2b β -lactamases were strongly inhibited, with measured K_i values in the nanomolar to picomolar range. The one group 2c enzyme tested was also strongly inhibited. However, the group 2d enzyme OXA-2 was not inhibited, and only some of the group 2a enzymes were inhibited, whereas others were unaffected. Furthermore, in some species such as *Staphylococcus aureus* and *Bacillus cereus* the group 2a β -lactamases exhibited a variation in their sensitivity to inhibition by BLIP from one strain to the other: strain ATCC1-27348 of *B. cereus* was inhibited ($K_i = 1 \text{ } \mu\text{M}$), whereas strain NCIB8933 was unaffected by BLIP at micromolar concentrations. In all cases the stoichiometry of BLIP binding to the enzymes is strictly 1:1.

The plasmid-mediated β -lactamase TEM-1 from the Gram-negative *Escherichia coli* is strongly inhibited by BLIP ($K_i = 0.6 \text{ nM}$). TEM-1 is the most clinically widespread of the class A



and the MIR phases showed clear electron density for 8 strands of β -sheet. The molecular model of BLIP was completed in two rounds of electron density fitting using the combined phases from MIR and calculated phases from the partial model (program SIGMAA²⁰). The initial refinement of the molecular structure was done with XPLOR²¹ and completed with PROLSQ²². The final refined crystal structure of BLIP based on data collected from a second native crystal (R_{merge} on intensity = 0.041, with data 89% complete to 2.1 Å resolution) consists of 1,235 protein atoms and 98 solvent molecules. The agreement factor ($R = \sum ||F_o| - |F_c|| / \sum |F_o|$) is 0.178 for 8,329 reflections in the range 20.0 Å to 2.1 Å resolution, with $|F_o| \geq \sigma |F_c|$ and good stereochemistry (r.m.s. deviations bond lengths, 0.017 Å; bond angles, 1.9° ; planar groups, 0.03 Å). There are only 3 residues (Ala 118, Tyr 50 and Arg 144) that are in high-energy locations on a ϕ , ψ plot, all of them in loop regions of very high B -factors and weak electron density. The surface loop from Asp 133 to Gly 145 is especially poorly resolved, with little or no density for the main-chain and side-chain atoms of Asp 136 to Arg 144.

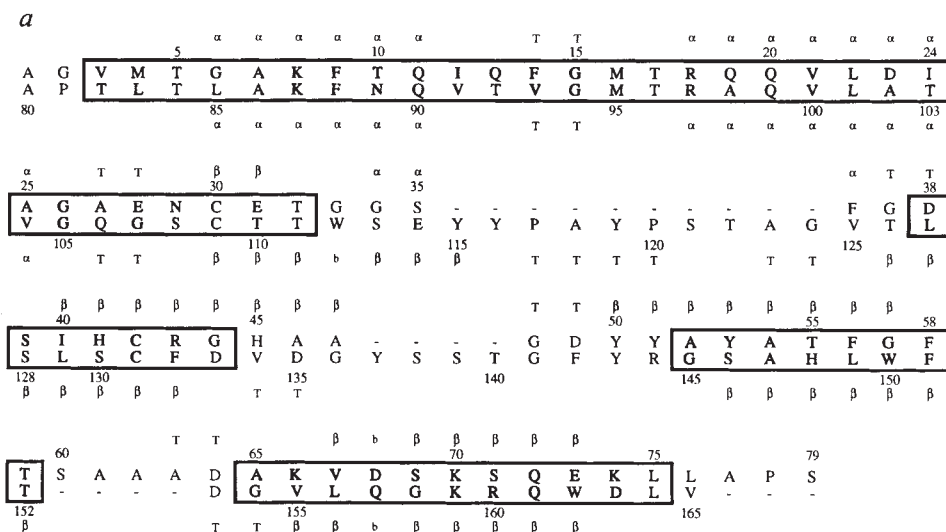


FIG. 2 *a*, Sequence comparison of the N- and C-terminal domains of BLIP. Regions of topographical equivalence are boxed. The secondary structure of each residue is given above or below the one-letter code for the amino acid (α for α -helical (or 3_{10} helix for Gly 34 – Ph 36), β for residues in β -sheet conformation, and T for residues that are part of reverse turns). Strands are numbered sequentially from the N terminus. The definition of the secondary structural features was taken from the program DSSP³⁷. *b*, Stereoview of the superposition of the Ca atoms of the two domains of BLIP. The N-terminal domain is represented by thick bonds between Ca atoms, the C-terminal domain by thin bonds. The topographically equivalent residues are shown in the boxed regions in *a*. When the two domains are superimposed by a least squares method, there are 56 topographically equivalent residues, for which the r.m.s. deviation is 0.87 Å for the 224 main-chain atoms (0.76 Å for the 56 Ca atoms alone).

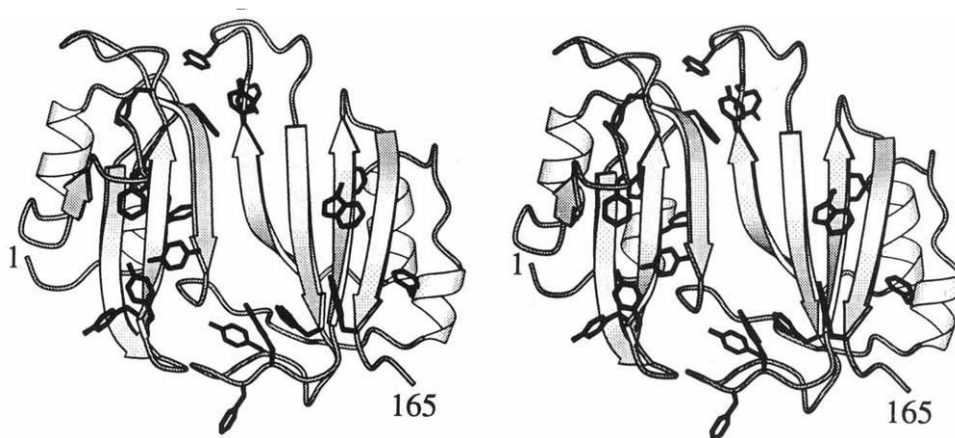
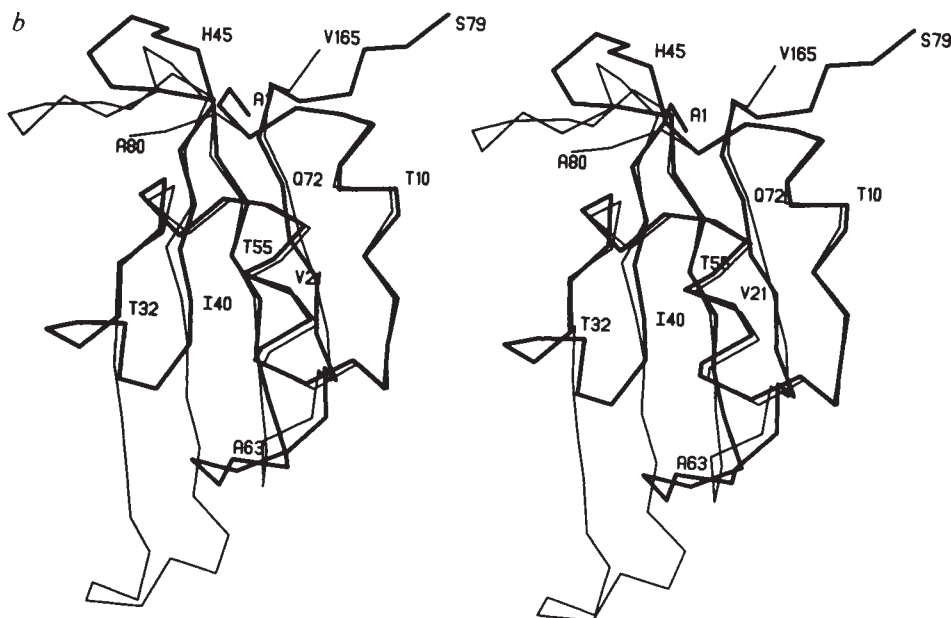


FIG. 3 Stereo diagram of Co-representation of BLIP viewed roughly perpendicular to the directions of the strands of the β -sheet. For clarity, only the side chains of aromatic residues are shown. The concave surface of the sheet is the location of most of the aromatic residues (13 on the concave side, 4 on the convex side). Some of the phenylalanine residues are located in the hydrophobic cores of the domains on the convex side of the sheet.

penicillinases and has recently caused further concern in that newly isolated variants of the enzyme have expanded their substrate preferences to third-generation cephalosporins⁶. Comparison of the structure of TEM-1 (refs 7, 8) with those of other known chromosomally encoded β -lactamases, PC-1 from *S. aureus*⁹ and 749/c from *B. licheniformis*¹⁰, indicates that although the overall topology is the same, there are regions of deletions and insertions as well as shifted secondary structural units that lead to dramatically different conformations in some of the surface loops. These differences are reflected in the relatively large r.m.s. deviations in positions of the 1,024 main-chain atoms (~ 2.4 Å) when the enzymes are compared in a pairwise fashion. It is likely that these structural differences affect the ability of a relatively large inhibitor such as BLIP to interact with the various β -lactamases (Table 1). On the other hand, comparison of the active sites of TEM-1 and the *S. aureus* and *B. licheniformis* β -lactamases indicates that the disposition of essential catalytic residues is very similar⁷. The overall r.m.s. deviations for the 76 common main- and side-chain atoms comprising the active sites of these enzymes are in the range 0.40 to 0.45 Å. Thus there is a high probability that the structural features of BLIP that specifically interact with and inhibit the active site region of TEM-1 will also be useful in the design of inhibitors for β -lactamases from other species.

Table 1 indicates that the natural targets of β -lactam antibiotics, the enzymes responsible for the synthesis and maintenance of the bacterial cell wall, are not generally inhibited by BLIP. The only penicillin-binding protein that was inhibited by BLIP ($K_i = 12 \mu\text{M}$) was PBP5 from *Enterococcus faecalis*; all other PBPs or DD-carboxypeptidases (see Table 1 legend) tested were unaffected. Although these data are preliminary, the insensitivity of PBPs to BLIP suggests that the natural role of BLIP is to protect the β -lactam antibiotics produced by *S. clavuligerus*, rather than to regulate cell wall morphogenesis.

How does BLIP inhibit β -lactamase activity? The molecule has an overall discoid shape and the 8-stranded β -sheet is markedly curved, with the helices packed on the convex side (Fig. 1a). These features combine to give BLIP a very similar topography to that of the TATA-box-binding protein^{11,12}. In considering how BLIP might interact with β -lactamases to inhibit them, we have noted a remarkable asymmetry of residues with aromatic side chains, with 13 of the 23 aromatic side chains being located on the concave side of the 8-stranded β -sheet (Fig. 3). These 13 residues in BLIP are largely exposed to solvent at this concave surface and so this might be the site of interaction with the β -lactamases. Alternatively, several prominent loops on the surface of BLIP may serve, as in the case of the serine-proteinase inhibitor family¹³, to interact specifically within the active site of the β -lactamases. It is not yet clear why BLIP should have evolved two similar structural domains when it interacts with 1:1 stoichiometry with its target enzyme. This is a novel feature for an enzyme-inhibitor protein. In the case of the serine proteinases, several protein inhibitors with duplicated domains exist¹⁴, but each inhibitor molecule can interact with several distinct enzyme targets simultaneously by means of surface loops. Modelling using the coordinates of BLIP and TEM-1 (ref. 7) will test the efficacy of currently used computer algorithms for docking molecules. We are determining the crystal structure of the 1:1 molecular complex of BLIP with TEM-1 at 2.8 Å resolution, which will be available to evaluate the correctness of the predicted modes of binding. □

7. Strynadka, N. C. J. et al. *Nature* **359**, 700–705 (1992).
8. Jelsch, C., Mourey, C., Masson, J.-M. & Samama, J.-P. *Prot. Struct. Func. Genet.* **16**, 364–383 (1993).
9. Herzberg, O. *J. molec. Biol.* **217**, 701–719 (1991).
10. Knox, J. R. & Moews, P. C. *J. molec. Biol.* **220**, 435–455 (1991).
11. Kim, Y., Geiger, J. H., Hahn, S. & Sigler, P. B. *Nature* **365**, 512–520 (1993).
12. Kim, J. C., Nikolov, D. B. & Burley, S. K. *Nature* **365**, 520–527 (1993).
13. Read, R. J. & James, M. N. G. in *Proteinase Inhibitors* Ch. 5 (eds Barrett, A. J. & Salvensen, G.) 301–336 (Elsevier, Amsterdam, 1986).
14. Laskowski, M. & Kato, I. A. *Rev. Biochem.* **49**, 593–626 (1980).
15. Bush, K. *Antimicrob. Agents Chemother.* **33**, 264–271 (1989).
16. Frère, J.-M., Leyh-Bouille, M., Ghuyssen, J.-M., Nieto, M. & Perkins, H. R. *Meth. Enzym.* **45**, 610–636 (1976).
17. Kabsch, W. & Sanders C. *Biopolymers* **22**, 2577–2637 (1983).
18. Hamlin, R. *Meth. Enzym.* **114**, 416–452 (1985).
19. Howard, A. J., Nielsen, C. & Xuong, N. H. *Meth. Enzym.* **114**, 452–472 (1985).
20. Read, R. J. *Acta crystallogr.* **A42**, 140–149 (1986).
21. Brünger, A. T. *XPLOR Manual, Version 1.5* (Yale Univ., New Haven, 1987).
22. Hendrickson, W. A. *Meth. Enzym.* **115**, 252–270 (1985).

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Structure of restriction endonuclease *Bam*HI and its relationship to *Eco*RI

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TYPE II restriction endonucleases are characterized by the remarkable specificity with which they cleave specific DNA sequences. Surprisingly, their protein sequences are in most cases unrelated, and no recurring structural motif has yet been identified^{1,2}. We have determined the structure of restriction endonuclease *Bam*HI at 1.95 Å resolution. *Bam*HI shows striking resemblance to the structure of endonuclease *Eco*RI (refs 3, 4), despite the lack of sequence similarity between them. We also observe some curious differences between the two structures, and propose an evolutionary scheme that may explain them. The active site of *Bam*HI is structurally similar to the active sites of *Eco*RI and *Eco*RV (ref. 5), but the mechanism by which *Bam*HI activates a water molecule for nucleophilic attack may be different.

*Bam*HI binds as a dimer to the symmetrical DNA sequence 5'-GGATCC-3', which differs from the *Eco*RI recognition sequence 5'-GAATTC-3' by only one base pair in each half site. Both enzymes cleave DNA at identical positions, following the 5'G on each strand. The structure of *Bam*HI was solved by combining phase information derived from multiwavelength X-ray data by algebraic⁶ and maximum likelihood methods⁷ (manuscript in preparation). The refined *R*-factor is 19.0% for a model consisting of 200 out of 213 amino acids and 116 water molecules. The dominant feature of *Bam*HI subunit structure is a large six-stranded mixed β -sheet sandwiched on both sides by α -helices. The dimer structure, generated by applying crystallographic two-fold symmetry, reveals a large cleft that could accommodate B-form DNA (Fig. 1).

*Bam*HI shows striking resemblance to the structure of *Eco*RI (refs 3, 4). This was unexpected because of the lack of amino-acid sequence correspondence between them. Both proteins are α/β structures, built around a central mixed β -sheet with α -helices surrounding it (Fig. 2). Many of the secondary structural

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1. Neu, H. C. *Science* **257**, 1064–1072 (1992).
2. Reading, C. & Cole, M. *Antimicrob. Agents Chemother.* **11**, 852–857 (1977).
3. Reading, C. & Hepburn, P. *Biochem. J.* **179**, 67–76 (1979).
4. Blazquez, J., Baquero, M.-F., Canton, R., Alos, R. & Bagnew, F. *Antimicrob. Agents Chemother.* **37**, 2059–2067 (1993).
5. Doran, J. C., Leskiw, B. K., Aippersbach, S. & Jensen, S. E. *J. Bact.* **172**, 4904–4918 (1990).
6. Sougakoff, W., Goussard, S., Gerbaud, G. & Courvalin, P. *Rev. Infect. Dis.* **10**, 879–884 (1988).

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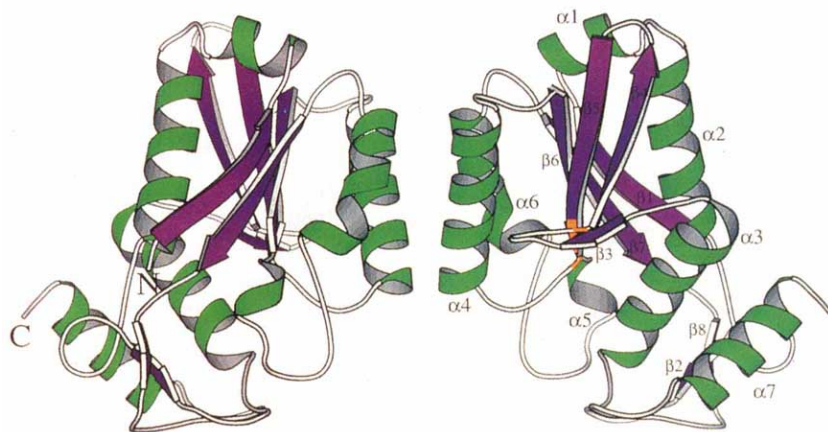


FIG. 1 A ribbon representation of the *Bam*HI dimer generated using the program MOLSCRIPT¹⁸. The twofold axis runs vertically in the plane of the page. The secondary structural elements have been labelled on one subunit. The N and C termini have been labelled on the twofold related subunit. α -Helices are coloured in green and β -strands in purple. The putative DNA-binding cleft is at the bottom of the dimer. The active site is indicated in yellow on one subunit only. Residues 79–91 are missing from the refined model and are therefore not shown. A main-chain connection between amino-acid residues 78 and 92 is shown to aid tracing of the polypeptide chain.

elements are arranged similarly in the two enzymes. In particular, a substructure consisting of five β -strands and two α -helices, comprising the β -meander and the Rossman fold in the two enzymes, can be superimposed with an overall r.m.s. error of ~ 2 Å (Fig. 2). We refer to this substructure as the common core motif (CCM). The CCM contains practically all of the active site, DNA cleft, and dimer interface residues. The active sites of both enzymes are located at the end of the β -meander and superimpose particularly well. The identification of a common core motif in *Bam*HI and *Eco*RI also helps to establish the probable identity of helices α_4 and α_6 in *Bam*HI as the counterparts of the inner (α_4) and outer (α_5) recognition helices of *Eco*RI (Fig. 2). In both enzymes, the two helices are situated close to and roughly parallel to the dyad axis. By pairing with the corresponding helices from the twofold related monomer, a dimer interface consisting of a bundle of four parallel helices is formed in both enzymes. The N termini of these helices are directed towards the major groove of the DNA in the *Eco*RI complex^{3,4}, and correspondingly towards the inferred DNA-binding cleft in the *Bam*HI dimer. It is interesting that based on our superposition, Arg 122 in *Bam*HI appears to be equivalent to the DNA recognition residue Arg 145 in *Eco*RI (Fig. 2). In this case, hydrogen bonds to the N7 atoms of the dinucleotide steps A-A in the *Eco*RI DNA site may be replaced by similar bonds to the G-A steps in the case of the *Bam*HI-DNA complex.

Beyond these similarities, the *Bam*HI structure reveals some striking differences from the *Eco*RI model^{3,4}. Overall, *Bam*HI is a much more compact enzyme than *Eco*RI. It lacks many of the secondary structural elements that interrupt the common core motif in *Eco*RI (Fig. 2). The most visible omissions are the inner and outer arms that precede the inner and outer recognition helices of *Eco*RI. These arms traverse the major groove of the *Eco*RI DNA, giving it a 'surrounded' appearance⁴. The beginning portion of the inner arm, described as the extended chain motif in *Eco*RI, also appears to be absent in *Bam*HI. As this section of the chain (Met 137–Gly 140) is considered to make an important series of contacts with the two outer pyrimidines by *Eco*RI, contacts to the corresponding pyrimidines in the *Bam*HI DNA site may be missing. The superposition of *Bam*HI and *Eco*RI structures also reveals some interesting differences outside of their common core motifs. Helices α_3 and α_7 in *Bam*HI appear to be analogous to helices α_2 and α_1 in *Eco*RI, but are shifted towards the DNA-binding cleft (Fig. 2). This results in only partial coincidence of the helices. Moreover, helix α_7 of *Bam*HI also seems to be antiparallel to helix α_1 of *Eco*RI. The most striking case of oppositely oriented helices, however, are helix α_2 of *Bam*HI and helix α_6 of *Eco*RI, which otherwise display almost perfect spatial overlap (Fig. 2a). The N and C termini of the two proteins also appear, curiously, to be switched with respect to the common core motif (Fig. 2). There may be more

than one explanation for these differences. We discuss later the possibility that the secondary structural elements of the two proteins may have topologically rearranged during the course of evolution.

Type II restriction endonucleases require only Mg^{2+} as a cofactor to catalyse the hydrolysis DNA phosphodiester, leaving free 5' phosphate and 3' hydroxyl groups^{1,2}. The reaction is considered to proceed by an in-line displacement of the 3' leaving group, in which an activated water molecule acts as the attacking nucleophile^{8,9}. A comparison of *Eco*RV and *Eco*RI structures shows them to be unrelated apart from a striking similarity in their active sites⁵. The active site of *Bam*HI also appears to be structurally conserved with that of *Eco*RI and *Eco*RV. Residues Asp 94, Glu 111 and Glu 113 in *Bam*HI are arranged almost identically to the active-site residues Asp 91, Glu 111 and Lys 113 in *Eco*RI, and residues Asp 74, Asp 90 and Lys 92 in *Eco*RV (Fig. 3). The *Bam*HI residues are three of the four amino acids identified as catalytically important from genetically isolated mutants of *Bam*HI that lacked cleavage activity but retained the ability to bind DNA^{10,17}. The structure of *Eco*RV bound to its cognate DNA site suggests that residues Asp 74 and Asp 90 of the enzyme, together with the reactive phosphate group of the DNA, help to coordinate a Mg^{2+} at the active site by providing an oxygen ligand each^{5,11}. Similarly, *Eco*RI cocrystals diffused with either $MgCl_2$ or $MnCl_2$ reveal a divalent cation in between the reactive phosphate and the *Eco*RI residue Glu 111 (ref. 4). Based on these analogies, it is a good guess that the role of residues Asp 94 and Glu 111 in *Bam*HI is also to help bind a Mg^{2+} at the active site, with one of the non-esterified oxygens of the reactive phosphate acting as the third ligand. The coordinated Mg^{2+} , in turn, may help to stabilize the negative charges on the pentavalent transition state. An important difference between the active site of *Bam*HI and those of *Eco*RI and *Eco*RV, appears to be the presence of Glu 113 in *Bam*HI at a position corresponding to Lys 113 in *Eco*RI and Lys 92 in *Eco*RV. There is a severe loss of cleavage activity, if either Glu 113 in *Bam*HI is substituted by a lysine residue¹⁰, or, conversely, Lys 92 in *Eco*RV is substituted by a glutamate residue¹¹. These differences in chemical identities of the residues may also intimate an important difference in the mechanism by which *Bam*HI activates a water molecule for the nucleophilic attack. Although the lysines in *Eco*RV and *Eco*RI appear to be well positioned, their high pK_a values would seem to preclude them from acting as general bases in order to deprotonate an attacking water molecule. In contrast, Glu 113 in *Bam*HI could deprotonate the attacking water molecule, and it may be the first example of a residue acting as a general base in restriction enzyme catalysis. For *Eco*RI and *Eco*RV, it has been suggested that a neighbouring phosphate group could activate the water molecule¹². Although the pK_a of a phosphate group would

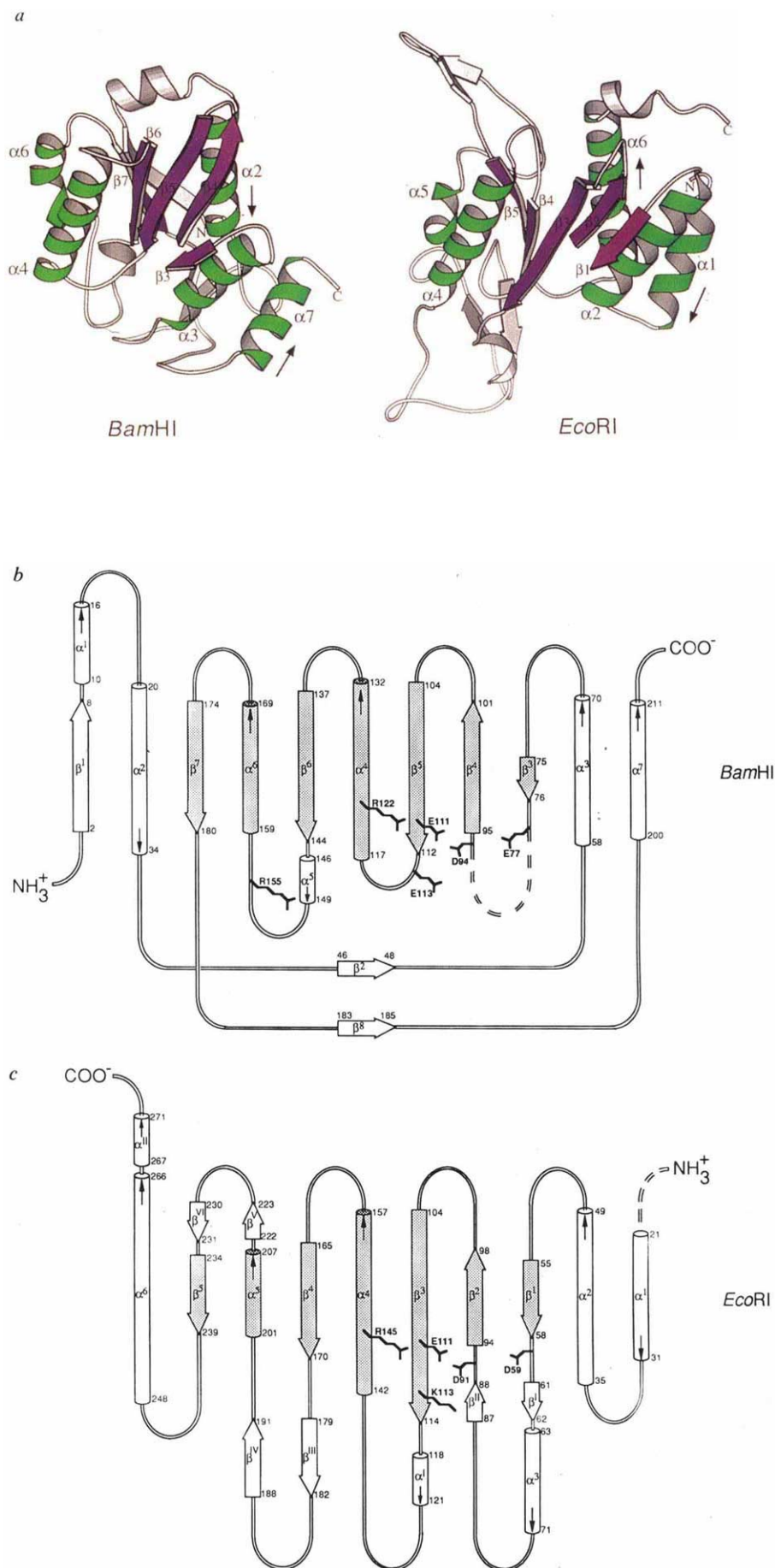


FIG. 2 *a*, Ribbon representation of *Bam*HI and *Eco*RI subunits in a similar orientation to Fig. 1. For clarity, regions 77–94 in *Bam*HI and 59–93 in *Eco*RI have been omitted. Common elements of secondary structure between the two enzymes are shown in colour. Arrows indicate the relative orientations of helices α_2 and α_7 in *Bam*HI, and α_6 and α_1 in *Eco*RI. Although these helices occur in similar positions in the 3-dimensional structures of *Bam*HI and *Eco*RI, their relative orientations have been reversed between the two structures. The secondary structures of *b*, *Bam*HI, and *c*, *Eco*RI indicate their similarity in the common core motif (shaded) and dissimilarity in the relative positions of the N and C termini. Secondary structural elements were defined using the hydrogen-bonding criteria in ref. 16. α -Helices are shown as cylinders and β -strands as broad arrows. Small arrows indicate the direction of the helices. In *Bam*HI, the secondary structure labelling is as for Fig. 1, and in *Eco*RI the labelling is as in ref. 4. The extent of the secondary structural element is indicated by residue numbers. Common elements of secondary structure between the two enzymes are shown in similar position in the two figures. Missing regions from each of the two structures are indicated as dashed lines. In *Bam*HI, from 79 to 91 inclusive, and in *Eco*RI, from 1 to 16 inclusive. In *Bam*HI, residues thought to be involved in the active site (Glu 77, Asp 94, Glu 111 and Glu 113) and in DNA binding (Arg 122 and Arg 155) are shown. Equivalent residues in *Eco*RI for activity (Asp 59, Asp 91, Glu 111 and Lys 113) and DNA binding (Arg 145) are shown.

appear to be too low to accept a proton⁵, recent studies with modified oligodeoxynucleotides appear to support this mechanism¹³.

What is the evolutionary relationship between *Bam*HI and *Eco*RI? The presence of analogous secondary structural elements at similar positions in the tertiary structure but at different locations in the primary sequence (Fig. 2) would appear to favour a relationship based on convergent evolution¹⁴. On closer inspection, however, it is possible to devise a divergent evolutionary scheme (Fig. 4) that could lead to the occurrence of N and C termini at opposite ends of the conserved core in *Bam*HI and *Eco*RI, and the reversed orientations of analogous helices α_2 and α_6 in *Bam*HI and α_6 and α_1 in *Eco*RI. In this scheme, no major rearrangement of an ancestor gene would be required

beyond simple additions and deletions to derive the final *Bam*HI and *Eco*RI structures. Helices α_2 and α_6 in *Bam*HI and α_6 and α_1 in *Eco*RI do not carry any catalytic or potential DNA recognition residues, but instead appear to play a structural role in closing off the central hydrophobic β -sheet. It is possible that this scaffolding function could have evolved to be provided from opposite ends of the proteins in *Bam*HI and *Eco*RI. An interesting question is why the sequences of *Bam*HI and *Eco*RI should have diverged so much, considering that the two enzymes recognize DNA sequences that differ by only one base pair in each half-site, and cleave their DNA sites at identical positions. One explanation may simply be that the specificities of *Eco*RI and *Bam*HI cannot be interconverted by simple point mutations. The lack of inner and outer arms in *Bam*HI, and the generally

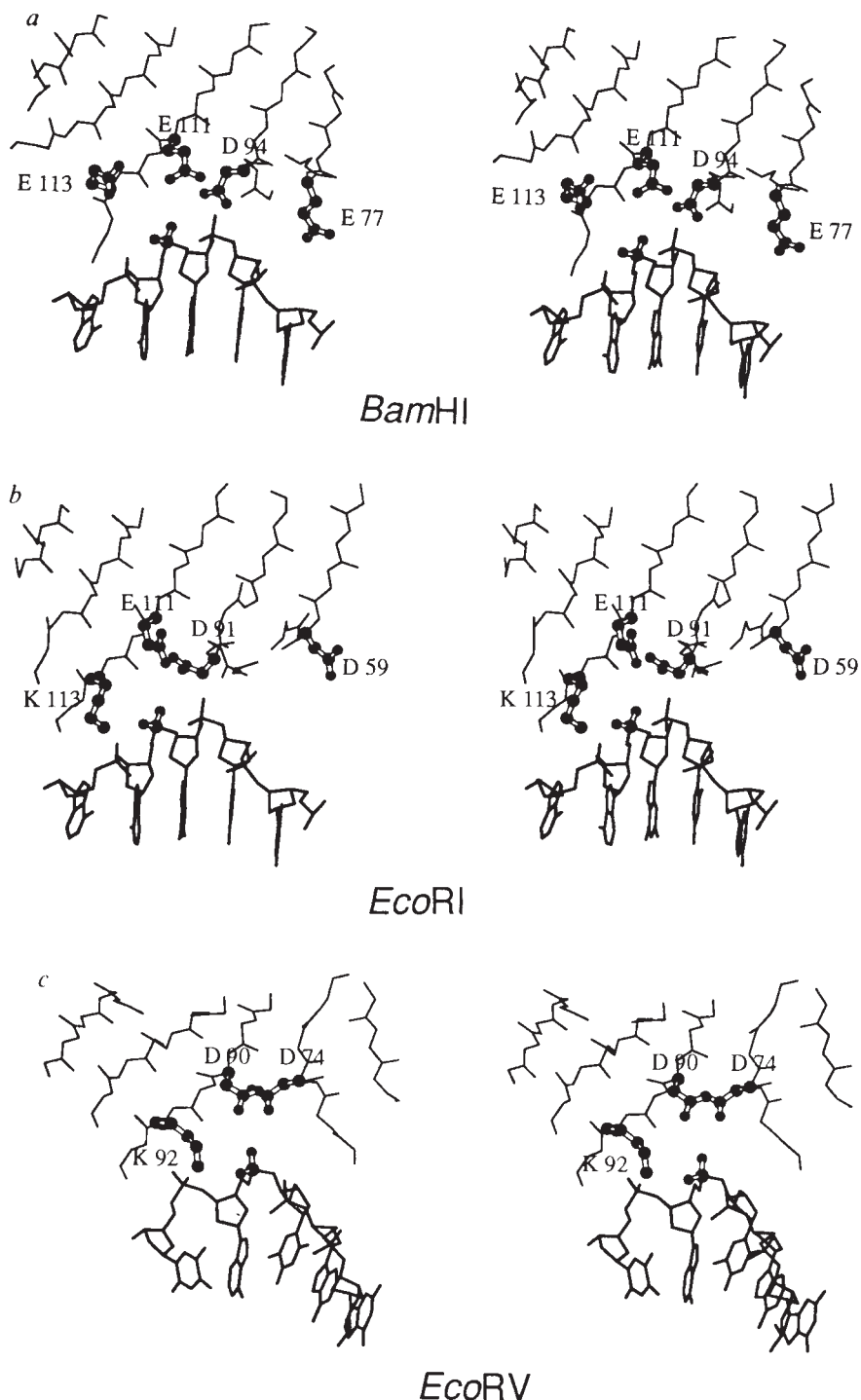


FIG. 3 Stereo views of the active sites of a, *Bam*HI; b, *Eco*RI; and c, *Eco*RV with DNA. Each view is in a similar orientation and shows equivalent regions of the structures. Active site residues and the reactive phosphate are shown in ball and stick representation. *Bam*HI DNA was obtained from the *Eco*RI-cognate DNA complex³ after a least-squares superposition of the enzymes.

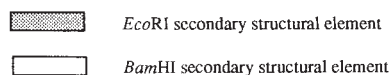
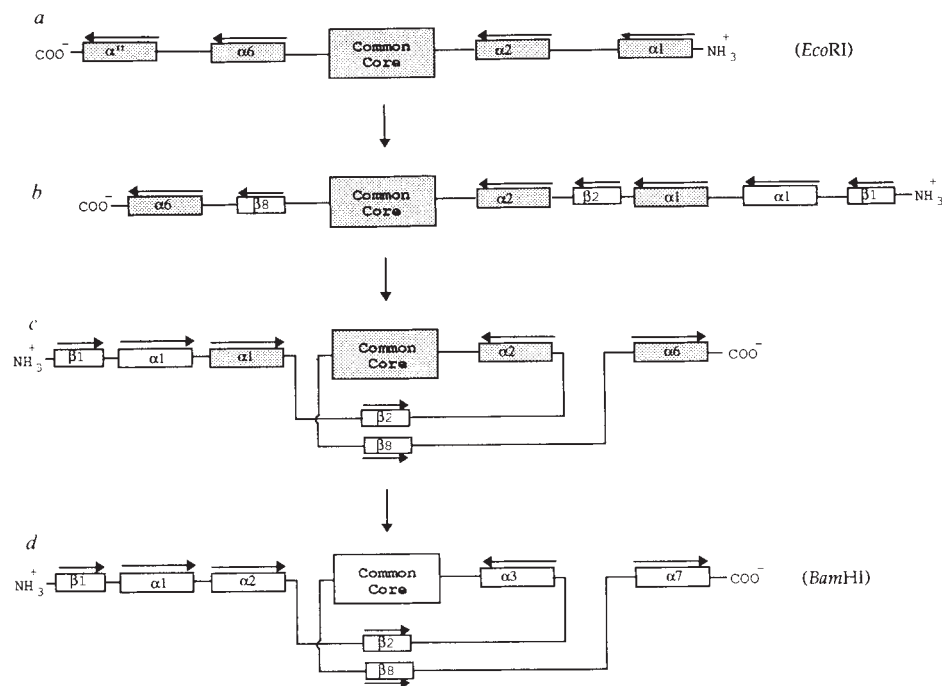


FIG. 4 An evolutionary scheme that may help to explain the topological relationship between the secondary structural elements outside of the conserved core in restriction enzymes *BamHI* and *EcoRI*. *a*, *EcoRI* structure consisting of four α -helices outside of the conserved core. The helices are labelled according to the *EcoRI* structure⁴. *b*, The addition and deletion of secondary structural elements during the course of evolution. The unshaded boxes are labelled according to the *BamHI* structure (compare Fig. 2*b*). *c*, Refolding of the protein such that the N and C termini swap their positions with respect to the conserved core. *d*, Labelling of the secondary structural elements according to the *BamHI* structure (compare Fig. 2). Note that helices α_2 and α_7 in *BamHI* are now in opposite orientations to their counterparts α_6 and α_1 in *EcoRI*.



unsuccessful attempts to alter the specificity of *EcoRI* (ref. 1) may be indications of the substantially different strategies by which the two enzymes dock to their DNA sites.

In *BamHI* and *EcoRI* dimers, the active sites are separated by ~17–19 Å along the DNA axis, whereas in *EcoRV* the distance is only ~2 Å. These distances are consistent with the cleavage properties of these enzymes. *BamHI* and *EcoRI* cleave their DNA recognition sequences at positions that are staggered by four base pairs (bp), producing 5' overhanging ends, whereas *EcoRV* cleaves its DNA site with a 0-bp stagger, producing blunt ends. The conformation of the common core motif in *BamHI* and *EcoRI* is ideally suited for the positioning of the two active sites at a relative separation of ~4 bp, spanning the major groove of DNA. In considering other restriction enzymes that might share structural similarity with *BamHI* and *EcoRI*, it is most likely to be those that also cleave their DNA sites with a (5') 4-bp stagger¹⁵. Nature of the cleavage pattern, rather than the actual DNA sequence recognized, may be the most important constraint on the overall conformation of restriction enzymes. □

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1. Heitman, J. in *Genetic Engineering* (ed. Setlow, J. K.) 57–107 (Plenum, New York, 1993).
2. Roberts, R. J. & Halford, S. E. in *Nucleases* (eds Linn, S. M., Lloyd, R. S. & Roberts, R.) (Cold Spring Harbor, New York, 1993).
3. Kim, Y., Grable, J. C., Choi, P. J., Greene, P. & Rosenberg, J. M. *Science* **249**, 1307–1309 (1990).
4. Rosenberg, J. M. *Curr. Opin. struct. Biol.* **1**, 104–113 (1991).
5. Winkler, F. K. et al. *EMBO J.* **12**, 1781–1795 (1993).
6. Hendrickson, W. A. *Trans. Am. Cryst. Assoc.* **21**, 11–22 (1985).
7. Otwinowski, Z. in *Isomorphous Replacement and Anomalous Scattering* (eds Wolf, W., Evans, P. R. & Leslie, A. G. W.) (SERC, Daresbury Laboratory, Warrington, UK, 1991).
8. Connolly, B. A., Eckstein, F. & Pingoud, A. *J. biol. Chem.* **259**, 10760–10763 (1984).
9. Grasby, J. A. & Connolly, B. A. *Biochemistry* **31**, 7855–7861 (1992).
10. Xu, S.-Y. & Schildkraut, I. *J. biol. Chem.* **266**, 4425–4429 (1991).
11. Selent, U. et al. *Biochemistry* **31**, 4808–4815 (1992).
12. Jeltsch, A., Alves, J., Maass, J. & Pingoud, A. *FEBS Lett.* **304**, 4–8 (1992).
13. Jeltsch, A., Jurgens, A., Wolfes, H., Maass, G. & Pingoud, A. *Proc. natn. Acad. Sci. U.S.A.* **90**, 8499–8503 (1993).
14. Creighton, R. E. *Protein: Structures and Molecular Properties* (Freeman, New York, 1993).
15. Anderson, J. E. *Curr. Opin. struct. Biol.* **3**, 24–30 (1993).
16. Kabsch, W. & Sander, C. *Biopolymers* **22**, 2577–2637 (1983).
17. Dörner, L. F. & Schildkraut, I. *Nucleic Acids Res.* **22**, 1068–1074 (1994).
18. Kraulis, P. J. *J. Appl. Crystallogr.* **24**, 946–950 (1991).

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CORRECTION

Formation of haematopoietic micro-environment and haematopoietic stem cells from single human bone marrow stem cells

Shiang Huang & Leon W. M. M. Terstappen

Nature **360**, 745–749 (1992)

WE retract the conclusion of this letter that a single cell can give rise to both a haematopoietic microenvironment and haematopoietic stem cells. Flaws in the material covered in the second paragraph of page 748 to the second paragraph on page 749 in particular lead us to misinterpret some of the critical results. Our subsequent attempts to confirm our key claim have been inconclusive. Cells depicted from the colonies attached to the complex bone marrow structures in Fig. 3*a* can give rise to cell colonies and round dispersed cells with a 'haematopoietic appearance', as shown in Fig. 4*a* and *c*, but immunohistochemical staining indicates that there is a large diversity of mesenchymal-derived cell types. □

ERRATUM

A convective model for the zonal jets in the atmospheres of Jupiter and Saturn

Scott A. Condie & Peter B. Rhines

Nature **367**, 711–713 (1994)

IN this letter a line of text was accidentally transposed: the first line of text in the left-hand column on page 713 ("columnar convection"^{18–20}). However, realistic estimates of...") should have appeared as the first line of text in the right-hand column on page 712. □

Righting an inherited wrong

A gene-therapy trial meets with success, and genetic mapping becomes an increasingly sophisticated but nonetheless accessible process.

THE April issue of *Nature Genetics* contains a landmark paper — the report by James Wilson and colleagues¹ at the University of Michigan Medical Center of gene therapy to treat a lethal condition, familial hypercholesterolaemia. Although this work comes more than ten years after the first (albeit unauthorized) human gene therapy procedures — carried out by Martin Cline in Italy and Israel — it is the first description of an extended follow-up period (18 months). The team concerned has documented, for the first time, the successful long-term correction of a genetic defect and the associated clinical improvement.

Familial hypercholesterolaemia is an inherited deficiency of the low-density lipoprotein (LDL) receptors which leads to dramatically increased levels of serum cholesterol and subsequent coronary artery disease. The female patient described in the article suffered a myocardial infarction at age 16, required a coronary artery bypass at 26, and did not respond to the cholesterol-lowering drug lovastatin. With a total serum cholesterol concentration of 545 mg dl⁻¹ (some five times greater than normal) and a ratio of LDL to high-density lipoprotein (HDL) cholesterol of around 11, she would have been at grave risk of further cardiac failure; moreover there were signs that one of her earlier bypass grafts had begun to deteriorate. But after gene therapy her LDL levels dropped by some 17 per cent, and the LDL:HDL ratio fell to just below 8 and is now responding to lovastatin.

There are a number of points that make Wilson and colleagues' account all the more intriguing. First, there is the sheer scale of the procedure. About one-fifth (250 g) of the patient's liver was removed and dissociated to yield 3.2×10^9 hepatocytes, which were plated out onto 800 cell-culture dishes. After a 12–18-hour exposure to recombinant retrovirus containing the LDL receptor gene, 150 ml of cell suspension was infused, through a catheter inserted in the mesenteric vein during the earlier surgery, back into the patient. As David Weatherall comments in an accompanying News and Views article², the procedure is "rather hair-raising".

The second point of interest is the overall inefficiency of the transfection process. Of the three billion liver cells removed for culture, only two billion survived, and of these only 20 per cent

showed any uptake of virus expressing the LDL receptor. A biopsy after four months suggested that as few as 1 in 1,000 to 1 in 10,000 liver cells expressed the transgene.

In all, these results give cause both for concern and for optimism. The worry lies in the overall inefficiency of this cumbersome *ex vivo* protocol, despite the opportunities it provides to manipulate and quantify the experimental procedure. This probably means that *in vivo* techniques — which would in some respects be preferable — are a long way off, given that they can be subject to far less control. The good news is that, for all the drawbacks of the process described in the paper, it worked — the patient's condition patently improved. Presumably even slight refinement of this first trial will yield even better results.

In a more immediate and less dramatic vein, two other reports in the same issue^{3,4} describe new tools to help generate meaningful genetic maps from the abundance of primary linkage data now being produced.

The first is an expert system computer program, MULTIMAP³. Based on a map-building algorithm, this program (available by sending email to multi-map@genome1.hgen.pitt.edu) aims to avoid some of the drudgery and associated errors that result from the many hours it takes to assemble a linkage map. There are also qualitative benefits. At present, when different groups choose to combine their 'local' maps it is done by comparing the various maps rather than the raw data; reworking the raw data would usually entail a computational and manual burden beyond the means of most groups. But MULTIMAP combines the heuristic aspects of previous map-building programs and a novel algorithm that sequentially builds a map from the primary data. The authors demonstrate the application of their program by producing, from scratch, a total linkage map of the human genome using genotypes of a total of 1,663 markers (data produced by Weissenbach *et al.*⁵, the NIH/CEPH Collaborative Mapping Group⁶ and six other smaller groups). The map, presented as a fold-out poster, offers nearly complete coverage of the autosomes with a sex-averaged map resolution of 6.2 centimorgans, using the 639 markers that could be allocated a unique map location with odds of greater than 1,000:1.

In the same vein, Kenneth Buetow and colleagues⁴ present an integrated genome-wide map, this time employing genotypes mostly generated by the National Center for Human Genome Research⁷ and Genethon⁵, complemented by many smaller data sets, and again using the CEPH reference panel. A program resembling MULTIMAP, but less fully automated, uses a new algorithm to assemble and integrate genotypes for more than 2,800 markers. The outcome is two sets of maps: high-confidence 'skeletal' maps, with an average density of 6.7 centimorgans, incorporating 544 simple tandem-repeat polymorphic markers; and annotated 'framework' maps representing the addition of a further 579 loci. Both maps and map-building software are available from linkage-server@chlc.org.

In the accompanying News and Views article⁸, Mark Lathrop discusses some of the steps involved in map construction and comments on the similarities between the two programs. Both build on the extensively tested CRI-MAP program⁹ and make extensive use of the option of checking the map for errors during its construction, thereby avoiding the repercussions of small initial errors that can lead to incorrect marker and locus orders.

Lathrop also points out that the groups have, most importantly, made sure that the software and data are available to others. This will allow individual groups to annotate and improve loci of local interest, and incorporate new markers as they become available, and enable the production of higher resolution maps which might prompt new investigations into mechanisms of interference, variable recombination rates and other parameters of the map. Such questions can be tackled with confidence only by using high-resolution, high-confidence maps.

Adrian J. Ivinson

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1. Grossman, M. *et al. Nature Genet.* **6**, 335–341 (1994).
2. Weatherall, D. *Nature Genet.* **6**, 325–326 (1994).
3. Matise, T.C., Perlman, M. & Chakravarti, A. *Nature Genet.* **6**, 384–390 (1994).
4. Buetow, K. H. *et al. Nature Genet.* **6**, 391–393 (1994).
5. Weissenbach, J. *et al. Nature* **359**, 794–801 (1992).
6. Cuttichia, A. *et al. Nucl. Acids Res.* **21**, 3003–3006 (1993).
7. NIH/CEPH Collaborative Mapping Group. *Science* **258**, 67–86 (1992).
8. Lathrop, G. M. *Nature Genet.* **6**, 326–328 (1994).
9. Lander, E. & Green, P. *Proc. natn. Acad. Sci. U.S.A.* **84**, 2363–2367 (1993).

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Catch and move — cut or fuse

Karin Schütze and Annette Clement-Sengewald

Still almost unbelievable, but true: light exerts force. With these forces it is indeed possible to catch and move cells or small particles and microsurgically to process them without any mechanical contact. As if by magic, objects are moved via focused laser light.

FOCUSED laser light is successfully used for 'non-contact', extremely precise, optical micromanipulation in biology, medicine and chemistry. The development of new optical techniques and the commercial availability of laser microscope systems with a wide variety of wavelengths and energies, have made powerful tools available to the scientists, opening new dimensions in cellular and subcellular research and applications.

Laser microbeam

The first such laser technique known as the 'laser microbeam' is an extension of work by Tschachotin¹ using a focused light source in a microscope as a tool for non-contact optical surgery. With laser microbeams a new era of micromanipulation has begun. Using an ultraviolet (UV)-laser microbeam, energy densities at the focal point are extremely high (up to 10^{12} W cm⁻²), so that almost all known materials can be microsurgically processed. As these energies arise only at the focal point, it is possible to work within the depth of a transparent object without opening it. Thus, holes of less than one micrometre in diameter can be drilled into cell membranes or membranes of cell organelles. Even rigid plant cell walls can be perforated without impairing cell viability. This was used, for example, to introduce genes into plant cells and organelles². Minute cuts through chromosomes *in vitro*³, as well as laser surgery on chromosomes *in vivo*⁴ became possible. Reversible destruction of cytoplasmic organization in Lilly pollen tubes has been demonstrated⁵. Drilling holes into contacting areas of cell membranes caused fusion of two adjacent cells in different species⁶ (for reviews see refs 7 and 8).

Optical tweezers trap

In 1987, another revolutionary laser tool, the so-called optical tweezers trap was developed for manipulation of cells and particles with a diameter of a few hundreds of nanometres up to several tens of micrometres⁹. The tweezers trap consists of a single strongly focused laser beam and is technically called a single-beam gradient force trap. It works by using radiation pressure forces and optical trapping principles developed in the 1970s. Optical tweezers have a number of unique capabilities. For example, with tweezers working in the near infrared, one is able to capture, move and position a wide variety of single cells and subcellular particles without direct contact

or significant optical damage. Thus, the torque of bacterial flagellae was determined¹⁰. Automated single cell sorting¹¹, studies of membrane formation and glycoprotein transport^{12,13}, as well as probing the elasticity of the cytoplasm¹⁴ and membrane skeleton¹⁵, have been described. In immunological studies, the reaction of killer cells, brought into close contact with their target cells, was investigated¹⁶. Chromosomes were optically trapped during mitosis in living cells without impairing their viability¹⁷. Also for the first time, it was possible to measure forces of motor molecules propelling organelles within a living organism¹⁸. *In vitro* experiments, using optical tweezers in combination with sophisticated devices to measure particle displacement from the trap centre, have resolved the details of motion and force generated by single motor molecules with nanometre and piconewton precision^{19–21} (for reviews see refs 22–25).

Combined microbeam and tweezers

Greulich's group in Heidelberg, Germany described the first combination of both laser systems in 1989. They used it for cutting and sorting chromosomes in molecular genetic studies^{26,27}. In 1991, Wiegand Steubing *et al.* at the Beckman Laser Institute in Irvine, California, used the 'cell fusion trap' to induce fusion of myeloma cells²⁸. One cell was brought into close contact with the target cell using optical tweezers. Several shots with the UV-microbeam at the membrane contact area caused fusion. Bern's group in Irvine also used the microbeam combination to cut chromosomes in living PtK₂ cells and to hold the fragments with the optical tweezers during mitosis²⁹. A Japanese group demonstrated three-dimensional optical trapping combined with laser ablation of polymer latex particles³⁰.

In 1993, an easy-to-handle, compact combination of UV-laser microbeam and optical tweezers trap with several, individually movable laser beams was introduced commercially. The PALM laser microscope system (P.A.L.M. GmbH, Wolfratshausen, Germany) combines the cutting abilities of the UV-laser microbeam with the manipulative abilities of optical tweezers. Both laser systems are coupled into a light microscope or laser scanning microscope (Zeiss, Oberkochen, Germany), still allowing fluorescence illumination or confocal viewing. The laser beams are focused to spot sizes of less than a micron in diameter. At the heart of the system is a special laser interface,

which allows focus adjustment independent of the microscope and three-dimensional beam positioning for each laser separately. Usually, laser beams are fixed at one position in the object plane and relative movement is performed by moving the background via the microscope stage. The unique feature of the PALM system is that all lasers can be used simultaneously and, in addition to the relative movement, can be moved independently from each other in all three dimensions. Thus *x,y* beam positioning within a circle of about 75 µm in diameter is possible. Furthermore, the infrared-laser is divided into two individually movable trapping beams to yield a double-beam optical tweezers trap. Another important point for nanometre accuracy in micromanipulation procedures is the possibility individually to correct laser focus positions in *z*-direction. This is especially necessary if the laser beam has passed through a thicker specimen, as any material within the light path behaves as a refracting medium and affects laser beam alignment. Beam positioning is motorized and comfortably managed via a joystick. The system is built modularly, so that each component is available separately and can be supplemented at any time. For minute object positioning the microscope is equipped with a multifunctional motorized *x,y,z* stage, which may also be used to semi-automate certain micromanipulation procedures. The system is connected to a CCD-camera with contrast enhancement and an image processor. Experiments are recorded on video and slides or prints can easily be retrieved from the videotape with a special photo-documentation unit.

Double-beam optical tweezers

The independent beam positioning of the PALM laser microscope system is particularly advantageous if objects need to be stretched out, aligned or turned around with two grabbing beams (for example, see Fig. 1a–d). Earlier this year, Finer *et al.* reported that they had used two optical traps to manipulate an actin filament through attached latex beads²¹.

Chu and colleagues have tested the recoil behaviour and visco-elastic properties of DNA molecules with a double-beam optical tweezers trap. For conformational studies they stretched out and fixed the elongated molecules to allow viewing with scanning tunnel or atomic force microscopy³¹. With the combined UV-laser microbeam and optical tweezers, chromosomes aligned be-

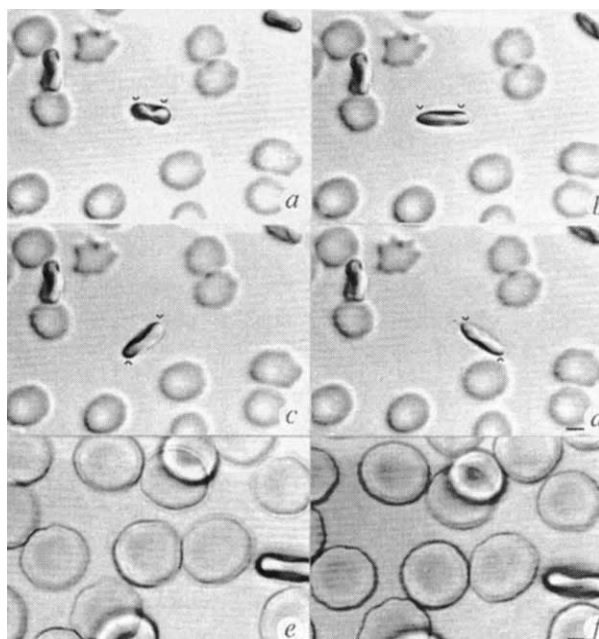


FIG. 1 Human erythrocytes *a*, are stretched *b*, and turned *c* and *d*, using two independently movable trapping beams (scale bar, 3 μ m). Tiny holes are poked into the membrane without causing leakage of haemoglobin *e* and *f*. Scale bar, 1.5 μ m.

tween the two trapping beams can now be cut at desired positions. Or cells can be microsurgically processed, while being held and positioned by the trapping beams. The ability to focus the laser beam precisely in the *z*-direction allows small holes to be poked, for example, in membranes of erythrocytes while they are trapped, without causing leakage of haemoglobin (Fig. 1*e* and *f*).

Furthermore, single cells or larger particles can be grabbed with two beams and rotated for optimal viewing to provide, for example, new insight into surface pattern configurations or membrane elasticity. With the combination of three independently movable laser beams it is also possible to induce fusion of cells, which are free floating in

suspension, and/or of cells with unequal sizes (Fig. 2).

Micromanipulation of gametes

An advantage of these new non-contact laser techniques is that they can be used in closed, sterile, temperature-controlled chambers free of evaporation and mechanical contact. There is no need for preparing and sterilizing microtools like holding pipettes or microneedles. Thus, complete sterility and high precision as well as selectivity can be achieved in micromanipulation procedures. These techniques may serve to supplement or replace the more standard manipulation techniques based on fine glass needles or pipettes mounted on mechanical micromanipulators.

An important application of combined usage of tweezers and UV-laser microbeam is the micromanipulation of gametes and early embryos for studying the fertilization process and possibly increasing the efficiency of *in vitro* fertilization^{32,33}. The UV-laser microbeam was successfully used to fuse blastomeres of mouse two-cell-stage embryos, which were capable of cell cleavage and *in vitro* development³⁴. Drilling holes into the zona pellucida of oocytes can be performed with high precision³⁵. Ng *et al.* succeeded in a significantly increased fertilization rate in mice

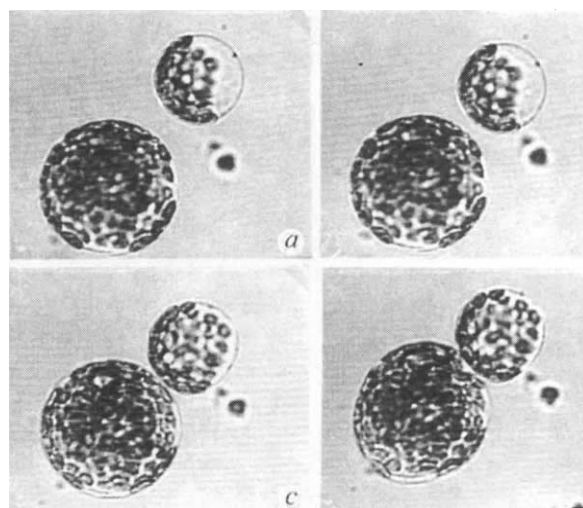


FIG. 2 *a* and *b*, Tobacco mesophyll protoplasts (courtesy of Professor H. U. Koop, Botanical Institute, University of Munich) are brought into close contact using optical tweezers. *c*, With the double-beam tweezers trap the cells are held in tight contact during UV-microbeam treatment to avoid scattering of the fusion partners due to laser light pressure. The two trapping beams are positioned at a distance of about 1 μ m right and left of the UV-laser site. *d*, In this way, fusion of free-floating cells may be induced. Scale bar, 6 μ m.

with live-born offspring after non-contact laser zona drilling^{36,37}. They also achieved increased hatching rates (S. C. Ng, personal communication). Optical trapping and relative force measurements of human sperm in a two-dimensional laser trap was reported by Tadir³⁸. Colon *et al.* caught human sperm in a three-dimensional laser trap and examined the influence of the trapping beam on sperm velocity³⁹.

The combination of UV-laser microbeam and optical tweezers for assisted fertilization was suggested by Ng *et al.* in 1992 (ref. 40). Using the PALM laser microscope system, we succeeded for the first time in drilling a hole into the zona pellucida and inserting a single sperm through the laser drilled hole into the perivitelline space⁴¹ (Fig. 3). The sperm could even be dragged around within the perivitelline space and brought into close contact with the oolemma to facilitate oocyte-sperm fusion. Fertilization was achieved in cattle with a rate of about 4 per cent and first treatment in humans with severe male infertility revealed about 20 per cent cleaved embryos.

The UV-laser can also be used to paralyse a sperm caught in the optical trap. One single UV-shot in close vicinity of the waving sperm stops movement immediately. Depending on the energy, motility is restored after some time. But with a precisely aimed second laser shot, the tail can be entirely separated from the sperm head. In this way, immobilized sperm or sperm without any or a short piece of the tail can be inserted underneath the zona pellucida. This procedure not only reduces the laser power needed for trapping but also gives a way of evaluating the

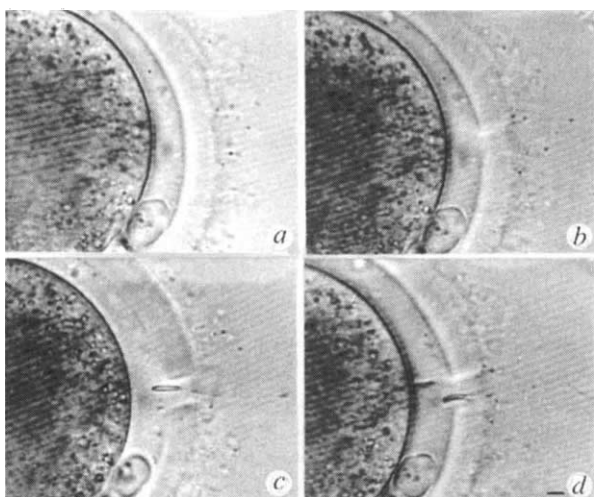


FIG. 3 Cattle oocyte *a*, before and *b*, after laser zona drilling. *c*, Sperm caught in the tweezers trap and dragged through the laser-drilled hole into the perivitelline space. *d*, The sperm is brought into close contact with the oolemma and a second sperm is inserted. Scale bar, 7 μ m.

necessity of the sperm tail for the fertilization process.

Future applications

The combination of a UV-laser microbeam and a double-beam optical tweezers trap with independently movable laser beams opens a new field of 'non-contact' micro-manipulation with novel applications in cell biology, medicine and chemistry. Due to the convenience and easy handling of the PALM laser microscope system, this quick and reliable tool can be used by almost anyone without dealing with the details of laser

physics. The ability to control the motorized microscope stage with a personal computer suggests the possibility of automated manipulation of bacteria, cells or gametes. The development of detectors capable of locating the position of trapped particles with nanometre accuracy, as well as the use of fluorescent molecule markers and use of specific antibodies attached to trapped microparticles, all indicate that laser manipulation techniques will play an increasing role in what is generally referred to as nanotechnology. □

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1. Tschachotin, S. *Biol. Centralblatt* **32**, 623-630 (1912).
2. Weber, G. *et al. Physiol. Plant.* **79**, 190-193 (1990).
3. Monajembashi, S. *et al. Exp. Cell Res.* **167**, 262-265 (1986).
4. Berns, M. W. *et al. Science* **213**, 505-513 (1981).
5. Schütze, K. *et al. Ber. Bunsenges. phys. Chem.* **93**, 249-252 (1989).
6. Wiegand, R. *et al. J. Cell Science* **88**, 145-149 (1987).
7. Berns, M. W., Wright, W. H. & Wiegand Steubing, R. *Int Rev Cytol.* **129**, 1-44 (1991).
8. Weber, G. & Greulich, K. O. *Int. Rev. Cytol.* **133**, 1-41 (1992).
9. Ashkin, A., Dziedzic, J. M. & Yamane, T. *Nature* **330**, 769-771 (1987).
10. Block, S. M., Blair, D. F. & Berg, H. C. *Nature* **338**, 514-517 (1989).
11. Bulcan, T. N. *et al. Appl. Opt.* **26**, 5311-5316 (1987).
12. Kucik, D. F. *et al. J. Cell Biol.* **114**, 1029-1036 (1991).
13. Edidin, M., Kuo, S. C. & Sheets, M. P. *Science* **254**, 1379-1382 (1991).
14. Ashkin, A. & Dziedzic, J. M. *Proc. natn. Acad. Sci. U.S.A.* **86**, 7914-7918 (1989).
15. Svoboda, K. *et al. Biophys. J.* **63**, 784-793 (1992).
16. Seeger, S. *et al. Cytometry* **12**, 497-504 (1991).
17. Liang, H. *et al. Exp. Cell Res.* **197**, 21-35 (1991).
18. Ashkin, A. *et al. Nature* **348**, 346-348 (1990).
19. Kuo, S. C. & Sheetz, M. P. *Science* **260**, 232-234 (1993).
20. Svoboda, K. *et al. Nature* **365**, 721-727 (1993).
21. Finer, J. T., Simmons, R. M. & Spudis, J. A. *Nature* **368**, 113-119 (1994).
22. Block, S. M. *Nature* **360**, 493-495 (1992).
23. Kuo, S. & Sheetz, M. P. *Trends Cell Biol.* **2**, 116-118 (1992).
24. Simmons, R. M. & Finer, J. T. *Current Biol.* **3**, 309-311 (1993).
25. Svoboda, K. & Block, S. M. *Ann. Rev. Biophys. Biomol. Struct.* **23**, 247-285 (1994).
26. Greulich, K. O. *et al. Labor 2000*, 36-46 (1989).
27. Greulich, K. O. & Weber, G. *J. Microsc.* **167**, 127-151 (1992).
28. Wiegand Steubing, R. *et al. Cytometry* **12**, 505-510 (1991).
29. Liang, H. *et al. Exp. Cell Res.* **204**, 110-120 (1993).
30. Misawa, H. *et al. J. appl. Phys.* **70**, 3829-3836 (1991).
31. Chu, S. *Science* **253**, 861-866 (1991).
32. Tadir, Y. *et al. Hum. Reprod.* **6**, 1011-1016 (1991).
33. Tadir, Y. *et al. J. Assist. Repr. Genet.* **10**, 121-125 (1993).
34. Clement-Sengewald, A. *et al. Proc. int. Soc. Opt. Eng.* **1876**, 187-194 (1993).
35. Neev, J. *et al. J. Assist. Repr. Genet.* **9**, 513-523 (1992).
36. Ng, S. C. *et al. J. Assist. Repr. Genet.* **10**, 204 (1993).
37. Liow, S. L. *et al. Theriogenology* **41**, 239 (1994).
38. Tadir, Y. *et al. Fert. Steril.* **53**, 944-947 (1990).
39. Colon, J. M. *et al. Fert. Steril.* **57**, 695-698 (1992).
40. Ng, S. C. *et al. J. Assist. Repr. Genet.* **9**, 191-196 (1992).
41. Schütze, K., Clement-Sengewald, A. & Ashkin, A. *Fert. Steril.* **61**, 783-786 (1994).

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From the analytical arena

Exhibits at Analytica '94, to be held next week in Munich, Germany, will include a range of particle characterization products, an enzyme immunoassay for the detection of autoantibodies to p53 and a second-generation mass analyser.

SIGMASTAT for Windows available from Jandel Scientific is a **statistical software package** that was designed specifically with the scientific and engineering research community in mind (*Reader Service No. 101*). The US\$495 program's procedures include descriptive statistics, *t*-tests, analysis of variance, correlation, *z*-tests, chi-square tests, nonparametric methods, linear, multiple linear and nonlinear regressions, power and sample size. If a researcher is unsure about

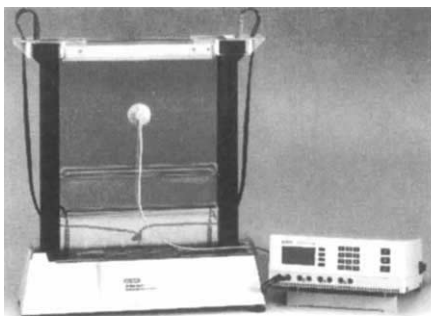
which test to use, SigmaStat's "Advisor" feature can be used. The program is also said automatically to handle missing and unbalanced data without making the user throw away any data. Data import options include text, spreadsheet (Lotus 1-2-3, Symphony, Excel and Quattro), database (dBase), SigmaPlot (Windows, DOS and Macintosh) and SigmaStat (DOS) files. The data worksheet stores over 1 billion data points and allows the user to transpose, insert, delete, sort, index and stack data. A range of user-defined mathematical transform functions are included — trigonometric, numeric, range and accumulation functions. 'Quick Transforms' allow the user to transform columns of data in one step.

Janssen Chimica is offering the research community very pure prototypical members of the **fullerene family** (*Reader Service No. 102*). The line-up includes fullerene C₆₀ (99.9 per cent plus purity, HPLC), fullerene C₆₀ (99.9 per cent), fullerene C₇₀ (99.9 plus per cent, HPLC) and fullerene C₇₀ (97 per cent). Also available are calixarenes — macro-

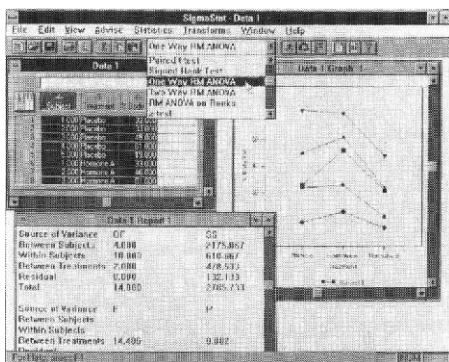
cycles made up of phenolic units meta-linked by methylene bridges and possessing basket-shaped cavities.

Molecular means

For all those **nucleic acid electrophoresis** applications, especially sequencing and single-strand conformation polymorphism (SSCP), which are sensitive to temperature fluctuations, Bio-Rad recommends its new Sequi-Gen II/PowerPac 3000 systems



For temperature-sensitive applications — see Bio-Rad.



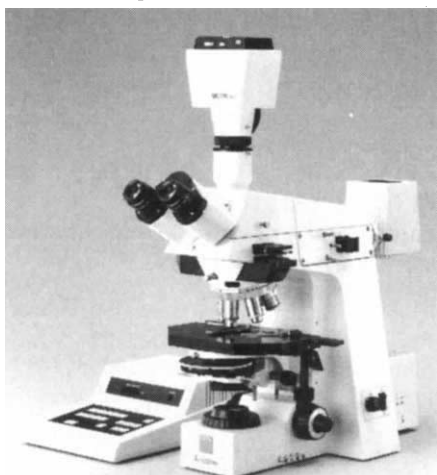
Statistical solutions from Jandel Scientific.

(*Reader Service No. 103*). The PowerPac 3000 features a temperature probe that monitors and controls run temperature by automatically adjusting power. When used together, the system can be used for a number of temperature-sensitive applications, including DNA sequencing, SSCP, microsatellite analysis, DNA footprinting, RNase protection assays, S1 nuclease mapping, primer extension analysis, mutation screening and oligonucleotide analysis.

Whereas 3–5 units of other polymerases are required for a standard primer extension reaction, Biometra says that with PrimeZyme, its new **thermostable DNA polymerase**, 1 unit or less of PrimeZyme will suffice (*Reader Service No. 104*). Derived from *Thermus brockianus*, PrimeZyme is supplied free of contaminating exo- and endonucleases, and is said to be thermostable — even after 2.5 h at 96 °C the remaining polymerase is decreased to only 50 per cent. Using the reaction and optimization buffers provided, primer extensions to fragments of 6,000 bases are said to be possible.

■ Assorted hardware

Easy operation and electronically controlled camera functions are features of the new MC 100 Spot **microscope camera** from Carl Zeiss (*Reader Service No. 105*). Despite the automation, all exposure parameters can also be individually selected for difficult specimens. To allow optimum exposure and imaging of specimens with especially interesting structures, the camera permits exposure metering both over the entire image field (centre-weighted averaging) and of details (spot metering). If the overall



Picture perfect — see Carl Zeiss.

image is of interest, the company says that the user should opt for centre-weighted averaging; spot metering covers a 3 per cent measuring area in the centre of the film format and ensures correct exposure times, even of small objects with a black background (as is often the case with fluorescence and darkfield) or a white background (brightfield).

The new Walk-ID **portable identification system** that makes it possible to read and print out bar codes on the spot has been developed by Tecan for clinical and other applications (*Reader Service No. 106*). Powered by rechargeable NiCd batteries, the



Tecan's lightweight labelling system.

system is designed to save time and money, and eliminate the kind of error that can arise with manual data transcription. Data input is via the barcode reader, memory card, magnetic badge, keyboard or serial interface. It can be shown on the display, stored on the memory card, transferred via the serial interface to another computer, and printed on the optional barcode printer. The optional software development kit allows the creation of application software in a program language similar to BASIC on any IBM or compatible PC. These applications can be downloaded to the device via the interface to facilitate standard data input/output procedures.

Products for **particle characterization** on show for the first time at Analytica on the Malvern Instruments stand will include the Mastersizer Micro and Zetamaster with an autotitrator accessory — enabling zeta potential to be measured in association with pH (*Reader Service No. 107*). The Mastersizer Micro's new single lens arrangement, enables the system to measure suspended particles from a range of 0.3 to 300 µm. The autotitrator accessory available with the Zetamaster enables automatic analysis of zeta potential versus pH. Windows software, installed on the Zetamaster, produces both a timetable of the results and a graph of zeta potential versus pH. The user can also establish the volume of titrant required to bring about the point of zero zeta potential.

■ Immunoreagents

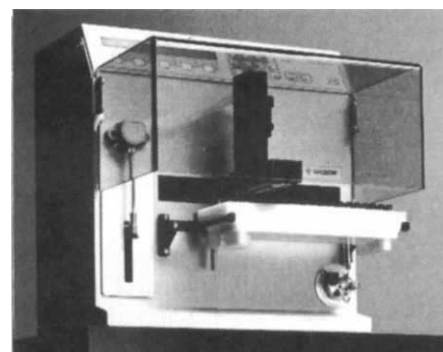
Genzyme Diagnostics will launching its **direct assay to measure low density lipoprotein (LDL)** at Analytica '94 (*Reader Service No. 108*). The kit utilizes a cocktail of polyclonal antibodies selectively to re-

move the other cholesterol particles, for example HDL and VLDL, from the sample. LDL can then be determined using any standardized cholesterol assay. Genzyme says that, unlike the Friedewald method, the direct LDL assay is unaffected by high triglyceride levels so there is no longer the need for patients to fast for 12 h before the sample is collected.

Dianova has a new enzyme immunoassay for the **detection of human autoantibodies against p53** (*Reader Service No. 109*). The company says that anti-p53 serum antibodies can be readily detected in 10–30 per cent (depending on the tumour type) of tumour patients using its ELISA procedure which is based on recombinant p53 antigen. The test has been designed for easy integration into a clinical laboratory set-up, providing an additional diagnostic tool in the follow-up and management of oncological patients. The assay uses precoated microwell strips and requires an incubation of 2 h 10 min.

■ Dispensers

The new Gilson 234 **autoinjector** can be used to dilute samples or standards, mix an internal standard with samples, aspirate, dispense and inject samples, reagents and standards (*Reader Service No. 110*). Five injection methods are offered: simple injection, external standard with and without dilution, and internal standard with and without dilu-



Gilson's autoinjector offers users a choice of five injection methods.

tion. The robot has an injection volume range of 1 to 500 µl, depending on the size of the injection loop. Users have the option of using total, partial or sample-saving, centred loop-filling techniques. Ten rack options are offered. The instrument is controlled via an integral command keypad through which users select the injection method, loop-filling technique, flow rates, number of injections per vial, number of samples, rinsing volumes, priority analysis, audit trail and coordination with other instruments. Up to 20 files may be stored. The instrument can be interfaced for automatic coordination with other devices in one of two ways: through the input/output contacts on the rear panel or via the Gilson serial input/output channel and the company's HPLC system controller software.

The Finnpiptette BioControl **trigger-activated multichannel pipette** from Lab-systems is designed to combine the feel of manual pipetting with an electronically-assisted pipetting action (*Reader Service No. 111*). The pipette's dual-stop trigger is said to increase comfort and virtually eliminate the risk of repetitive stress injuries. Both forward and reverse pipetting are possible without a separate programming step. Other design features include right- and left-handed tip ejection, an autoclavable tip cone module, a rapid recharge stand, and automatic cut-off and restart.

■ Spectroscopy

Lasermat 2000 is Finnigan Mat's second-generation **mass analyser** (*Reader Service No. 112*). Based on the earlier Lasermat version, this new machine offers improved performance, specially developed laser control software and automated autosampling capabilities. Lasermat 2000 incorporates a new detection system and faster electronics for signal digitization; this is designed to result in improved resolution and greater mass accuracy. The company has also developed algorithms to optimize the laser control parameters and further simplify hands-off operation. The incorporation of a 48-position autosampler allows unattended operation, which is particularly advantageous when dealing with large batches of samples, such as peptide fractions from an HPLC.

Varian's new Zeeman **graphite furnace atomic absorption spectrometer**, the SpectrAA-600 with Zeeman background correction, can be used to analyse up to 999 samples of up to 20 elements in a multi-element sequence with random access sampling (*Reader Service No. 113*). The automated four-lamp turret and automatic



Varian's SpectrAA-600 multi-tasking atomic absorption spectrometer.

wavelength and slit selection are designed to simplify instrument set-up and operation. Varian says that the instrument can complete two replicates in about a minute. The SpectrAA 600 has an IBM OS/2-based user interface. A 486 computer is integrated into the instrument's design. Sample preparation parameters — sample labels and weight and volume correction factors — can be uploaded directly from a floppy or network drive

before an analysis, and data can be downloaded to a network drive or LIMS system after an analysis.

A comprehensive range of Hitachi's **spectrophotometers** will be on show on the Colora Messtechnik stand at Analytica '94 (*Reader Service No. 114*). The display will run the whole gamut, covering fluorescence, UV/visible and atomic absorption instruments. Two fast-scanning fluorescence spectrophotometers will be on show: the F-2000 which is designed for the measurement of intracellular cation concentrations, and



Windows-controlled UV/visible spec.

the F-4500 which is suitable for applications ranging from fast three-dimensional scans for 'fingerprinting' mixtures or determining the purity of HPLC solvents, to fluorescence microtitre assays and rapid kinetics measurements. Both use Windows-based software. In the UV/visible line, Hitachi will be exhibiting its U-2000 spectrophotometer, which will be shown in conjunction with the AS-3000 autosampler. The Windows-controlled U-3000 for research applications features microfocused instrument optics, which can be used with microlitre-scale cuvettes. Completing the line-up is the Z-8200 series tandem flame/furnace atomic absorption spectrophotometer, which uses polarized Zeeman correction methods.

The new HP4500 **benchtop inductively coupled plasma mass spectrometer** (ICP-MS) from Hewlett-Packard is designed to allow detection of most elements, even including potassium, calcium and iron, down to the parts-per-trillion (p.p.t.) level (*Reader Service No. 115*). The Omega lens ion optics system bends the ion beam, which, HP says, allows the quadrupole and detector to be mounted off-axis and reduces background to less than 2 c.p.s. across the mass range. In addition, an interface known as 'Shield-



Hewlett-Packard's ICP-MS system — said to have detection limits in the p.p.t. range.

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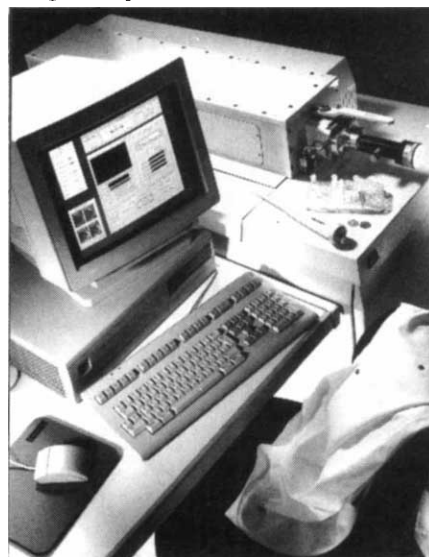
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Reader Service No.14

Torch' is intended to eliminate many polyatomic interferences, allowing potassium, calcium and iron to be determined at p.p.t. levels. Other design features include a compact, solid-state quadrupole radio-frequency generator operating at 3.0 MHz and a dynode detector with a dual-mode acquisition system giving a full eight orders of linear dynamic range. The system is controlled from an HP Vectra PC running Microsoft Windows-based ChemStation software. Applications can be run off-line during sample acquisition to maintain a high sample throughput. Custom reports can be generated as the system transfers data directly to the co-resident Microsoft Excel spreadsheet program.

New from Fisons Instruments is the ToFSpec family — a series of matrix-assisted, laser desorption ionization **time-of-flight mass spectrometers** (Reader Service No. 116). The VG ToFSpec-E and SE are designed to provide the biochemist with the



ToFSpec family of mass spectrometers.

tools for determining the molecular weights of peptide, protein and oligosaccharide mixtures. The company says that biopolymers up to 100,000 daltons in mass can be handled, and the analysis of sub-picomole amounts of sample takes a few seconds. Available in both linear and reflectron versions, the instruments enable the loading of up to 15 samples at a time onto a single slide. Despite occupying the same amount of floor space as the original VG ToFSpec, the VG ToFSpec-E offers a 50 per cent extension in flight path over its predecessor, with a corresponding increase in mass resolution; the SE offers a further 50 per cent extension. Both the E and SE models allow automatic programming of the source and reflectron voltages, thus allowing acquisition of post source decay product ion spectra. Additionally, an optional gas cell allows the generation of collision induced decomposition products, while the fast ion gate option enables precursor ion selection, thereby providing

full MS/MS capability. This year, Fisons Instruments is introducing a fully automated inlet system, available on both models, which will allow up to 40 samples to be loaded onto a single strip at one time. Alternatively, the inlet system may be extended to accommodate several 40-sample strips at once, or a single two-dimensional gel-plate blot. Each model is controlled from a common Windows-based data system, VG Opus.

■ Reagent round-up

Calbiochem-Novabiochem announces the release of its new 1994 **signal transduction catalogue** and reference guide (Reader Service No. 117). The product areas include antibodies to signalling proteins, calcium channel and metabolic modulators, calcium caged chelators and fluorescent probes, calmodulin and calmodulin inhibitors, enzymes and growth factors, enzyme activators, inositol phosphates, nitric oxide synthase inhibitors, protein kinase and protein phosphatase inhibitors, and protein tyrosine kinase inhibitors. As a reference guide, this catalogue includes molecular structures and formulas, protocols and application notes.



Off the shelf.

The new **ready-mixed stains** from Shandon Scientific, available from LSI, are designed to save time and money and provide consistent results (Reader Service No. 118). Based on the Perl's Prussian Blue technique, the new Rapid Chrome iron-staining kit comprises individual disposable dropper sticks that incorporate two separate crush-



Staining made simple — new from LSI.

able vials, each containing hydrochloric acid and potassium ferrocyanide. When required, the user need only squeeze the sticks in a premarked position, crushing the vials and mixing the two reagents. The cell nuclei can be counterstained with nuclear fast red, which is held in a separate dropper stick and released by crushing. For added flexibility, the alcohol-resistant nuclear fast red can also be supplied separately. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.